ADRENAL CORTEX

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JOSIAH MACY, JR FOUNDATION CONFERENCE PROGRAM

I RANK FREMONT SMITH
Medical Director

To THOSE of you who are guests I want to say Welcome and to those of you who attended last year s Conference in addition to Welcome I want to say how delighted we are to have you back

I would like to give you as briefly as possible an outline of what we hope to accomplish by these conferences As most of you know they are an attempt to provide an opportunity which is not ordinarily available for multiprofessional communication around a given topic—in this case the adrenal cortex Although the fertility of the multi discipline approach is recognized in principle universities scientific societies and journals have not yet made adequate provision for chan nels of interdisciplinary communication

The Foundation is interested in furthering knowledge about the adrenal cortex but it is interested also in the broad aspects of the problems of communication and integration which are importan for the advancement of the whole of science We are trying to promote better recognition of the wholeness and unity of nature and to over come the artificial fragmentation of science which university depart mentalization almost inevitably produces

These conferences are not for the presentation of formal papers but for free and informal discussion of ideas concepts research plans and difficulties It is our belief that reports of research at scientific meetings and in the journals have been forced into a narrow mold in which logical sequence leading to inevitable conclusions has been substituted for the much more unpredictable processes by which scientific inquiry and the advance of knowledge actually take place This overemphasis on logic gives a false impression of the nature of the scientific process Logic is a very important aspect of science but equally essential is the *intuitive or creative aspect* In my opinion research is as creative as the painting of a portrait or the composition of a symphony The cre ative aspects of research flow largely from unconscious non rational processes logic is necessary to rearrange to test and to validate or invalidate We hope to give in the published transactions of our con ferences a clearer reflection of what takes place in the laboratory and what goes on in the minds of investigators than now appears in sci entific literature

Thirteen groups are now functioning under the Conference Program and cover the following topics Aging Biological Antioxidants Blood Clotting Blood Pressure Connective Tissues Consciousness Cyber

netics Infancy and Childhood, Liver Injury Metabolic Interrelations Nerve Impulse Renal Function and Adrenal Cortex

Each group holds annual two day meetings for a period of five years It is our belief that only through continued association in an atmosphere of friendliness and mutual confidence can effective communication (exchange of ideas data methods, and plans) across the barriers of the professions and specialties be promoted As a result of these meetings we have seen plans and ideas modified and conclusions more clearly specified or placed in a broader perspective Spontaneous collaboration often takes place between investigators working in different departments or in different universities

As a nucleus fifteen scientists comprise the original group of members for any conference These are selected by the Chairman of the Conference in consultation with the Foundation Every effort is made to include representatives from all pertinent disciplines From time to time new members are added by the group to fill gaps in viewpoint or technique A limited number of guests are invited to attend each meeting but for the purpose of promoting full participation of all members and guests attendance at any meeting is limited to twenty five It is inevitable with this limitation that we must exclude many key investigators in each of the specialties represented One of the difficulties in planning these conferences is the necessity to leave out so many scientists whom we would like to include

A point which I should like to stress before closing is that between the disciplines there are real difficulties in communication — partly emotional and partly semantic Emotionally some of us accept only data coming from those methods or disciplines with which we are familiar It is important that we do justice to the validity of data and methods from other disciplines On the semantic level the physical and biological sciences have little difficulty the medical psychiatric and social sciences can understand each other fairly well but to bridge the gap from the physical and biological sciences to the psychological and social is indeed difficult[1] Medicine which must be equally concerned with the psychological and social as with the biological and physical aspects of man provides the greatest opportunity as well as necessity for mutual understanding among representatives of all the sciences I believe that the hope for the unification of science lies in the development of a *Science of Man* in which medicine must play an integrating role

In closing I want to say that the Conference Program is an experiment and that you are part of that experiment In these conferences we hope that the spirit of free inquiry and critique so necessary to scientific progress will operate not only in promoting communication within the group but also in assisting the Foundation to [illegible]ve its conference procedure

INTRODUCTORY REMARKS

C N H LONG
Chairman

DR FREMONT SMITH has covered most of the points that I was going to emphasize among them the informality of these meetings and the fact that we do not expect the individuals who open the discussions to present us with formal papers in which all of the answers are neatly tied up with ribbons

Perhaps I should say that as Chairman I am responsible for the selection of the topics which will be discussed If any of you feel that there are others of greater importance we can consider them for an other year

Before proceeding with the program I should like to point out that the subject matter of this Conference is one very much in the public eye So much so that when my taxi driver this morning asked me where I was going and I replied To an adrenal conference he said You are going to talk about ACTH He also asked me if ACTH is all that it is cracked up to be So perhaps we may find out at this Conference whether ACTH is all it is cracked up to be

Dr Li will open the discussion by telling us about the chemistry of the adrenal cortical hormone

THE PRESENT STATUS OF THE CHEMISTRY OF ACTH

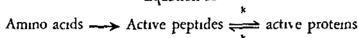
CHOH HAO LI

Department of Biochemistry University of California

TWO YEARS ago I presented a summary of the biochemistry of ACTH before the Seventeenth Macy Conference on Metabolic Aspects of Convalescence (1) Today I shall attempt to discuss certain new facts on the chemical nature of ACTH which have appeared since 1948

For a number of years I have been puzzled by the following questions How does a protein of molecular weight 20 000 or up permeate into a cell for action? Once injected into the body is the protein hormone degraded or changed into another form before it exerts its biological function? It seems reasonable to assume that the protein molecules dissociate or hydrolyze to smaller units and that one or more of these dissociated fragments are the real reactive units If this assumption is correct the following equilibria must exist inside the organism

Equation A



$$\text{Amino acids} \longrightarrow \text{Active peptides} \underset{k}{\overset{k}{\rightleftharpoons}} \text{active proteins}$$

Hence the biologically active peptides could be obtained from glandular extracts as well as from the hydrolysates of the active protein It should be pointed out that the occurrence of biologically active peptides depends upon the specific rates k_1 and k If $k_1 > k$ it would be impossible to discover active peptides in the gland Conversely if $k_1 < k$ both active peptide and protein are present

Recent investigations on ACTH suggest that the relationship noted in Equation A does exist in the extracts of anterior pituitary glands In their earlier works Anselmino *et al* (2 3) and Tyslowitz (4) claimed that ACTH activity could be ultrafiltered through a collodion or cellophane membrane Morris and co workers (5) have recently confirmed these observations In our laboratory (6) fractions (peptides?) from extracts of sheep gland which are soluble in 5 per cent trichloracetic acid have been shown to possess ACTH activity

Before the data concerning the hydrolysates of ACTH protein are discussed it is important to keep in mind that the hormone molecules

(protein or peptide) are not the same for different species The earlier physical data of Sayers *et al* (7) and Li *et al* (8) on pig and sheep hormone suggest the similarity of the protein preparations from these two species Our recent work and that of others show clearly that the *chemical* nature of the sheep and pig hormone is very different Furthermore, the hormone from ox glands behaves in yet another manner so that the method used to purify sheep hormone cannot be applied to the isolation of the hormone from ox extracts I I Geschwind in our laboratory has been preparing active extracts from pig glands by methods similar to that employed by Astwood and co workers but the same techniques are not applicable to ox pituitaries Our work since 1942 has been conducted solely with the sheep hormone, and it should be kept in mind that the following discussion is derived from the data obtained with sheep ACTH

In order to demonstrate the reversible reaction of active protein $\xrightarrow{k}$ active peptides of Equation A we have been investigating various

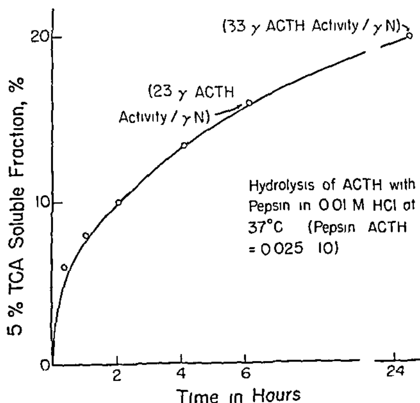


FIGURE 1 Hydrolysis of ACTH protein with crystalline pepsin Solvent 0 01 M HCl 1% hormone solution and 0 005% pepsin at 37 C

hydrolysates of the isolated ACTH protein It was found that trypsin chymotrypsin papain and alkaline digests of the protein hormone contain no activity Pepsin and hydrolysates on the other hand retain the biological potency

The course of hydrolysis with pepsin is shown in Figure 1 It is now well established that the pepsin digests of ACTH proteins (sheep and pig) possess adrenal stimulating activity The procedure for the preparation of ACTH peptide mixture presently employed in our laboratory is as follows

> One gram of ACTH and 25 mg crystalline pepsin were dissolved in 100 ml 0 052 M HCl the solution was kept at 37° C At the end of five hours 100 ml 10 per cent trichloracetic acid were added and the precipitate formed was centrifuged off The supernatant was extracted with ether to remove the trichloracetic acid The aqueous solution was then frozen and dried in vacuum

The peptide mixture thus obtained has an activity almost twice as great as the protein hormone as is shown in Table I The physicochemical properties of this peptide mixture are listed in Table II G Hess in our laboratory has demonstrated by the procedure previously employed by Fromageot *et al* that the active components in the pep

TABLE I

Bioassay of ACTH Fractions Before and After Pepsin Hydrolysis

Fraction	Dose (m o g m N)	Number of Rats	Average Depletion of Ascorbic Acid (mg /100 gm d n l)	ACTH Equivalent
				m gm
L2010M — before	0 75	19	—121 9 ± 17 5*	5 7
L2039M† — after	1 00	17	—142 2 ± 23 5	11 0

* M n ± stand d er o

†F ve pe c t t chl t d ol ble f ct on b f th t acs n

TABLE II

Certain Physicochemical Data of ACTH Peptide Mixture

Molecular Weight	1200
Diffusion Constant $D_{,} \times 10$	30 0
Sedimentation Constant S_{f}	0 45
Average Amino Acid Residues	8
N %	13 2
NH -N %	1 4

tide mixture are probably basic in nature The average molecular weight of the mixture was determined by Kai O Pedersen from ultracentrifuge and diffusion data (9) and was found to be 1200 It is interesting to note that Waugh (10) has reported the molecular weight of the active principle of Armour s ACTH to be in the vicinity of 2000

In a recent report (11) from the Armour group Lesh et al stated All of the results described indicate that the active moiety of the ACTH protein is of molecular size considerably greater than the ACTH peptides recently reported It is not unlikely that the activity of the mixture of peptides previously reported was due to the presence of a few per cent of a larger molecule having a very high biological potency It is due to this paper of Lesh *et al* that some doubts have been raised with regard to the molecular size of the ACTH active peptide(s)

For the past year we have been in close collaboration with Tiselius Pedersen and others at Uppsala and our existing data suggest that the average molecular weight of the peptide mixture cannot be too far from 1200 In fact we can separate the peptide mixture into five fractions containing different degrees of activity The average molecular weight of these fractions varies from 570 to 2600 (see Table III) One of the interesting facts deriving from the experiments was that the fraction having a molecular weight of 570 contains some ACTH activity It is quite possible that the ACTH peptides prepared from pig glands are different from those obtained from sheep pituitaries

The complexity of the peptide mixture is easily demonstrated by paper chromatography As shown in Figure 2 at least 6 ninhydrin reactive spots are identifiable when butanol 10 per cent acetic acid is

TABLE III

Approximate Average Molecular Weight of Various ACTH Peptide Fractions

Fraction	ACTH Activity	Concentration*	S_{20}	D_{20}	M †
			10'	10	
A	+	1 95	0 27	38	570
B	+	1 5	0 21	41	410
C	++	1 5	0 41	24	1400
D	+++	1 6	0 66	21	2500
E	+++	0 85	0 73	23	2600

*The solvent was 0 2 M NaCl

†The partial specific volume V_{20} was assumed to be 0 70

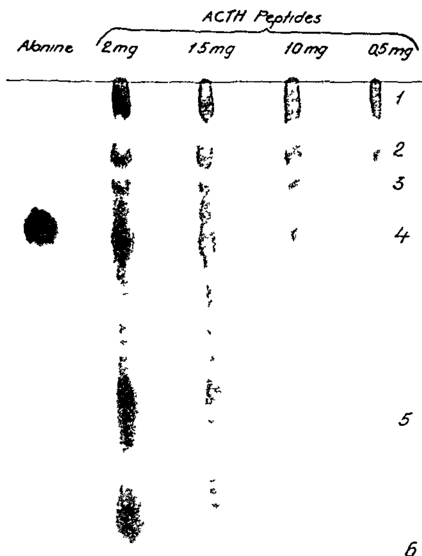


FIGURE 2 Different amounts of ACTH peptide mixtures (Preparation L 1669 CS) run on one dimensional paper (Whatman No 4) chromatogram solvent butanol 10% acetic acid

TABLE IV

Nitrogen and ACTH Activity Distribution in Different Spots of ACTH Peptides Obtained in Paper Chromatogram*

Spot No	N	Per Cent of Original Amount N	A corbic Acid Lowering per 100 gm Adrenal	ACTH Standard Equivalent
	mg	%	mg	mcgm
1	2 20	34 4	117 (6)†	5
2	0 40	6 3	1 (4)	0
3	0 23	3 6	5 (3)	<0 2
4	0 11	1 7	7 (4)	<0 2
5	0 11	1 7	0 (3)	0
6	0 23	3 6	33 (1)	0 3

Buta ol 10% acetic acid as the solvent 49 mg in 49 spots n th orig nal (see F gure 1)

†Number f rats in parentheses 1 mcgm N per 100 gm body w ght was i jected intra venously for the assay

employed as the solvent It was shown that most of the activity is in the immobile spot which contains approximately 35 per cent of the total original nitrogen Some activity was also found in spot No 6 (see Table IV) It is difficult to assume that the activity in this spot is due to the contamination of spot No 1 as these two spots are far apart In addition there are other ninhydrin positive areas between them and these areas have no activity Is more than one active peptide formed in the pepsin digest of ACTH protein?

Other chromatographic experiments indicate that more than one peptide component is indeed adrenocorticotropically active I will de scribe one of the experiments conducted by Dr Tiselius Dr Hogdahl and me *

> Two hundred and fifty milligrams of the peptide mixture were dissolved in 20 ml 0 1 M HCl and pressed into a charcoal column which was packed with the wet absorbent (Carboraffin Supra charcoal and Hyflo Super Cel in a ratio of 1 3) A solution of 1 per cent of Zephiran chloride in 0 10 M HCl was used as the displacer The column was driven with a pressure of approximately 1 kg per sq cm Fractions (0 5) were collected in test tubes using an automatic col lector apparatus A total of 312 fractions was obtained Micro Kjeldahl nitrogen and adrenal stimulating activity were determined in each tube

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U publ hed data

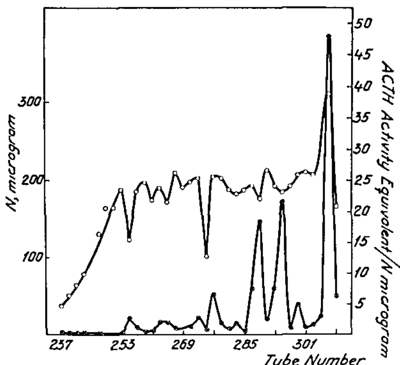


FIGURE 3 Displacement development of ACTH peptides (Preparation L 1669 CS) Two hundred fifty milligrams of the peptides were used displacer 1% Zephiran chloride adsorbent Carboraffin Supra and Hyflo Super Cel in a ratio of 1 3

The results are shown in Figure 3 It is evident that eight active peaks occur from Tube 253 to Tube 309 The highest activity was found in Tube 307 and this also had the highest concentration It should be noted that on nitrogen basis this fraction has a specific activity about ten times that of the starting material If the original peptide mixture contains only one active component one would expect that the ACTH activity would concentrate in a narrow displacement zone The fact that the biological activity spreads out in more than one fraction suggests that the original material must consist of several ACTH active peptides The present urgent needs of ACTH in clinical medicine demand the isolation of these active peptides in the hope that they may some day be synthesized in the laboratory

Let us now return to the discussion on the structure of the ACTH protein A complete amino acid analysis of the hormone has been obtained by the microbiological technique R Mendenhall in our lab

TABLE V

Composition of ACTH Protein (Molecular Weight 22 000)

Constituent	Gm /100 gm Protein	N as Per Cent of Protein N	Estimated Number of Residues
N	15 6		
S	2 8		
NH_3	1 0	5 3	
Arginine	8 7	17 9	11
Aspartic Acid	6 7	4 5	11
Cystine	8 2	6 1	8
Glutamic Acid	15 6	9 5	23
Glycine	8 0	9 6	23
Histidine	1 3	2 3	2
Isoleucine	3 1	2 1	5
Leucine	7 8	5 3	13
Lysine	5 0	6 1	8
Methionine	1 4	8 4	2
Phenylalanine	4 0	2 2	5
Proline	8 2	6 4	16
Serine	6 0	5 1	13
Threonine	3 2	2 4	6
Tyrosine	2 4	1 2	3
Valine	3 4	2 6	6
TOTAL		97 0	155

oratory is determining the composition by the starch column technique of Stein and Moore A comparison of the data obtained by these two methods should give us an indication of the existence of D amino acids The data in Table V do not reveal any special features different from ordinary protein Neither cysteine nor SH groups can be detected the amount of S in the protein is derived solely from the cystine and methionine content From the content of histidine methionine threonine tyrosine phenylalanine the *minimum molecular weight was computed* to be 22 000 (Table VI) Taking the molecular weight of 22 000 for the ACTH protein the molecule has 8 –S–S– bridges

R R Porter and I determined the number of end groups in the ACTH protein last year at Cambridge The method used was that of Sanger s dinitrofluorobenzene technique We have concluded tentatively that the molecule has only one terminal end group and that this group is alanine Since the protein molecule has 6 –S–S– bridges it must have a structure of a coil with an alanine residue in one end (See Figure 4)

TABLE VI

Minimum Molecular Weight of ACTH Protein

Amino Acids	Gm /100 gm Protein	Minimum Molecular Weight	Average No of Residues	Calculated Molecular Weight
Histidine	1 3	11 940	2	23 880
Methionine	1 4	10 660	2	21 320
Threonine	3 2	3 720	6	22 320
Tyrosine	2 4	7 550	3	22 650
Phenylalanine	4 0	4 130	5	20 650
	Mean			22 162

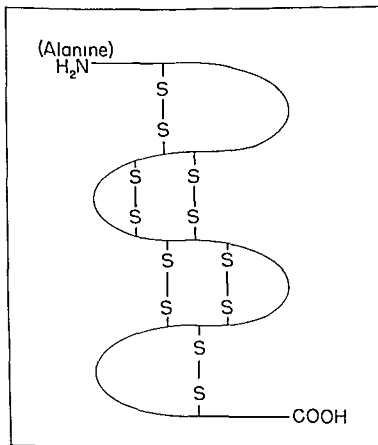


FIGURE 4 A diagram illustrating the structure of ACTH protein

We have shown earlier that acetylation of the free amino groups causes a loss of activity Recently J I Harris in our laboratory has confirmed the essentiality of free amino groups using other reagents One of our aims is to investigate the location of active peptide moieties in this coil chain Whether the active peptides reside in one of the two terminal ends or are concealed inside the core is of great theoretical interest

Some months ago we were able to show that the activity of ACTH protein is increased by heat in 0 025 M HCl solution (12) This observation is now extended to actual acid hydrolysis with 1 M HCl at 100° C Results are shown in Figure 5 and Table VII It is evident that the activity is retained even when the protein is hydrolyzed to an extent of 90 per cent If the 1 M HCl solution was kept at 100° C for only ten to twenty five minutes some activation occurred

From the foregoing discussion we can say with certainty that partial hydrolysis of ACTH protein with pepsin or acid does not destroy the activity and that the resultant active peptide mixture can be fractionated

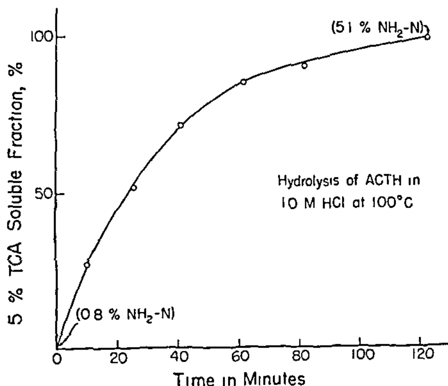


FIGURE 5 Hydrolysis of ACTH protein with acid Solvent 1 M HCl 1% hormone solution at 100 C

TABLE VII

Bioassay of Acid Hydrolysates* of ACTH Protein

Time	5% TCA Soluble Fraction†	Depletion of Ascorbic Acid at 1 mcgm Nitrogen Level	ACTH Activity Equivalent
m	per c n	mg/100 gm ad al	m gm
10	27	−134 −138 −125 −145 −142 −167	10 5
25	52	−195 −117 −149 −132 −111 −146	10 5
40	72	−129 −99 −57 −177 −67 −84 −75	2 5
80	91	−124 −82 −91	2 6
120	100	+35 +115 −9 +44 +27	0

1% sol tio of ACTH p ot i 1 M HCl t 100 C

†1 mcgm og n f the 5% TCA sol bl f ct on pe 100 gm body w ght of hypophysec t m l rat was u d

into more than one adrenocorticotropically active peptide Moreover the average molecular weight of the active peptide mixture is of the order 1000

Finally may I suggest a matter of terminology for this group to discuss? It is now an established fact that there are two types of ACTH active fractions one is protein and the other is peptide in nature The name ACTH should be reserved for the protein hormone as it has been used in the literature for the isolated hormone since 1942 Al though animal and human studies have so far not demonstrated differ ences between the protein and peptide hormone in the future it might turn out that biological action or activities of the two types of ACTH are not the same It appears to be of advantage to identify the peptide hormone with another name I would therefore suggest that the pep tide hormone be called ACTIDE

DISCUSSION

White Can you tell us what you think is the line of demarcation between *proteins and peptides?*

Li Perhaps the best criterion is the degree of dialyzability In many cases, the dialysates obtained from hydrolytic products of proteins are still precipitable with 5 per cent trichloracetic acid I would say that the solubility in 5 per cent trichloracetic acid is a better criterion in differentiating protein from peptides Materials that are not precipitable in 5 per cent trichloracetic acid may be taken as peptides

Loeb Dr Li is it your impression that the difference lies in the more reactive peptide group or in the inactive protein group?

Li As far as the protein is concerned the solubilities of beef sheep and pig behave very differently We have no experiences with the active peptides derived from pig or beef but reports in the literature indicate that they may be different chemical entities

Pincus The molecular weights are all about the same approximately 20 000?

Li Yes the molecular weight of sheep and pig ACTH protein is identical

Bloch If you test the material precipitated with trichloracetic acid does it have ACTH activity?

Li The trichloracetic acid precipitate of the partial hydrolysates is *practically devoid of ACTH activity*

Sayers Would you explain what you mean by 23 mcgm of ACTH activity? Is that in terms of standard?

Li The ACTH activity is read from a standard curve obtained from the assay of a highly purified ACTH preparation at different dose levels The standard is the sheep ACTH protein isolated by our published method (8)

Pincus How does your standard compare with La 1 A?

Li We obtained a La 1 A sample from Dr Munson four years ago and assayed it in hypophysectomized male rats using the Sayers procedure It was found that our purest preparation had activity almost identical with that of La 1 A I must admit that only three or four animals were used for each assay and the results should not be considered accurate At any rate it has higher potency at six hours hydrolysis At zero time the potency is only 7 mcgm ACTH per mcgm of nitrogen

Thorn Was there evidence of increased activity in some of the preparations that were not as purified as your original material?

Li No

Thorn I wonder if you were reconstructing activity that would be due to other factors in the purified material per mcgm of nitrogen Is it true that this material is as active to begin with as La 1 A and that a further increase in activity is observed with fractionation?

Li Yes The La 1 A molecule is different from the sheep hormone we have This is the important point to keep in mind

Astwood Do the amino nitrogen determinations indicate that on the average you have broken the molecule in half? If the molecular weight was 20 000 to begin with how then do you now have an average molecular weight of 1200?

Li This is a mixture some may have small molecular weight but the average value is 1200

Astwood I don t see why the amino nitrogen does not increase in proportion to the decrease in average molecular weight

Li It depends upon the structure of peptides in the mixture

Thorn Dr Astwood s question raises the problem of the real significance of the value of the average molecular weight

Astwood Of your values which do you regard as the more reliable the average molecular weight of 1200 or the value of 1 4 for the percentage of amino nitrogen?

Li There is no direct relationship between free amino nitrogen and average molecular weight of a peptide mixture The former depends upon the chemical constituents and the latter upon the size

White Have you done any other type of molecular weight estimations? The boundary with that size molecule must be very unsatisfactory in the centrifuge What does the boundary look like Dr Li? Is it very diffuse?

Li We have not carried out these determinations other than in the ultracentrifuge There is no sharp boundary but the colloids that migrated away from the meniscus could be easily located

Ralli Were the Armour group working with sheep pituitary?

Li No the Armour group has always worked with the pig glands

White What disturbs me particularly is the question of molecular weight in relation to free amino acid nitrogen Do you have any evidence of ring formation? Of possible cyclization of peptides?

Li We have no information concerning the cyclic nature of the peptide mixture

Astwood I would be very much interested to know the nature of the displacer employed

Li A detergent zephiran chloride—I got it from Sweden

Thorn We have gallons of it in our operating room It is a detergent used routinely in the preoperative preparation of the skin

Long Is 5 mcgm of your material equivalent to 1 mcgm of ACTH?

Li We assay on the nitrogen basis one mcgm nitrogen per 100 gm body weight

Long So 1 mcgm lowers the adrenal ascorbic acid 117 mg per cent

Li Yes

Long How many micrograms of nitrogen does Spot No 1 in Figure 2 represent—1 mcgm of ACTH?

Li Yes

Astwood That would mean that it was less active than La 1 A

Li Yes Slightly less active The La 1 A gives about 7 mcgm ACTH equivalent per 1 mcgm of nitrogen, whereas the material from Spot No 1 (see Figure 2) has an activity of 5 mcgm per 1 mcgm of nitrogen Our assay results should not be taken to be accurate but they do give us the location of ACTH activity during our fractionation We want to know only whether the activity is there or not We feel that it is not necessary to insist upon accuracy until we obtain a highly purified material I would like to ask Dr Sayers opinion on this

Sayers One dose level assays of this type will detect differences in potency of the order of five or ten times They may not demonstrate differences of two or even three times The evidence suggests that molecular weight does not influence the response of the adrenal cortex to ACTH It is by no means conclusive proof

Thorn Dr Sayers is the assay such that a molecule of 500 molecular weight would give a poor assay because of more rapid adsorption than a molecule of 2 000 molecular weight? In other words is the difference between these preparations a real difference in potency or a difference in activity based upon the relative rate of adsorption which is so important in bioassay of this type?

Sayers The slope of the log dose response curve is influenced by factors such as adsorption and synergism We have observed that the regression lines of large molecular weight ACTH and of small molecular weight ACTH have the same slope

Li How about contaminations does that affect the slope?

Sayers We have tested crude extracts of pituitary tissue which contain practically all the tropic hormones of the adenohypophysis and compared these crude extracts with highly purified preparations (highly purified in a biological sense) The slopes of the log dose response curves are not significantly different We have tentatively concluded that contaminants in the adenohypophysis do not influence the action of ACTH Remember in the ascorbic acid depletion test ACTH has a very short period of time to act namely one hour In assays of longer duration for example the adrenal weight technique may very well be

influenced by such contaminants as growth and thyrotropic hormones

Ralli Did you try any of the posterior pituitary hormone which might be a contaminant in some of these preparations?

Li We have not been investigating the posterior hypophyseal hormone contaminations in our peptide or protein preparation

Astwood Were you not surprised that with such a complex mixture you observed only two steps in the displacement chromatograms?

Li It depends upon the displacer as well as the nature of the adsorbent Nitrogen distribution does not indicate the number of components in the mixture it merely reflects the amount of material that has been displaced at a particular time

Astwood Would you not think that with a mixture of many substances you would have a series of many steps? If there were so many compounds present that the steps were obscured you would expect to have a progressively increasing concentration of material in the effluent

Li We should not confuse the displacement analysis with the well known Tiselius frontal analysis The steps appearing in the curve do not necessarily indicate a new component (Figure 3)

White The end group turns out to be alanine and you know that there is only one end group and one peptide chain?

Li Yes

Bloch What happens to the activity if you use an agent which reduces the disulfide bonds?

Li That is a problem we have been working on for some time Preliminary data indicate that reduction of –S–S– to –SH does not destroy the activity

Bloch You would expect an uncoiling after reduction of disulfide bridges

Li Yes but no loss of activity was observed

Thorn What is the initial assay of your starting material?

Li Seven micrograms ACTH equivalent per 1 mcgm nitrogen

Long Do you often observe a difference of plus 115 mg per cent between the two levels of adrenal ascorbic acid in the hypophysectomized animal?

Li This surprised us and I would like to have Dr Sayers comment

Sayers Once in a while you get a value that is quite a bit out of line I usually consider such a deviation to be due to a gross error in weighing or in ascorbic acid analysis Remember that the technique has a number of individual steps each of which is a possible source of error

Li Anyway there is no activity after two hours hydrolysis with 1 M HCl

Sayers Did you compare this with material that was not boiled

Long Is 5 mcgm of your material equivalent to 1 mcgm of ACTH?

Li We assay on the nitrogen basis one mcgm nitrogen per 100 gm body weight

Long So 1 mcgm lowers the adrenal ascorbic acid 117 mg per cent

Li Yes

Long How many micrograms of nitrogen does Spot No 1 in Figure 2 represent—1 mcgm of ACTH?

Li Yes

Astwood That would mean that it was less active than La 1 A

Li Yes Slightly less active The La 1 A gives about 7 mcgm ACTH equivalent per 1 mcgm of nitrogen whereas the material from Spot No 1 (see Figure 2) has an activity of 5 mcgm per 1 mcgm of nitrogen Our assay results should not be taken to be accurate but they do give us the location of ACTH activity during our fractionation We want to know only whether the activity is there or not We feel that it is not necessary to insist upon accuracy until we obtain a highly purified material I would like to ask Dr Sayers opinion on this

Sayers One dose level assays of this type will detect differences in potency of the order of five or ten times They may not demonstrate differences of two or even three times The evidence suggests that molecular weight does not influence the response of the adrenal cortex to ACTH It is by no means conclusive proof

Thorn Dr Sayers is the assay such that a molecule of 500 molecular weight would give a poor assay because of more rapid adsorption than a molecule of 2 000 molecular weight? In other words is the difference between these preparations a real difference in potency or a difference in activity based upon the relative rate of adsorption which is so important in bioassay of this type?

Sayers The slope of the log dose response curve is influenced by factors such as adsorption and synergism We have observed that the regression lines of large molecular weight ACTH and of small molecular weight ACTH have the same slope

Li How about contaminations does that affect the slope?

Sayers We have tested crude extracts of pituitary tissue which contain practically all the tropic hormones of the adenohypophysis and compared these crude extracts with highly purified preparations (highly purified in a biological sense) The slopes of the log dose response curves are not significantly different We have tentatively concluded that contaminants in the adenohypophysis do not influence the action of ACTH Remember in the ascorbic acid depletion test ACTH has a very short period of time to act namely one hour In assays of longer duration for example the adrenal weight technique may very well be

of ACTH provided by Armour I would like to show the results of assays on these preparations

Thorn We have never had a preparation comparable to the material prepared by eighty minutes of boiling

Ralli I don t know about ACTH but that will destroy definitely the antidiuretic activity of pitressin

Li You don t need eighty minutes twenty five minutes treatment will destroy practically all the antidiuretic factor

Long Do you infer that the biological activity of this peptide frac tion is different in any way from the whole protein?

Li We don t know yet Perhaps in the future some differences may be found Chemically they are different substances

Long I wanted to bring out the point that a different biological activity is associated with a difference in chemical structure

The subject of the fractionation of ACTH is still before us Perhaps Dr Astwood would care to resume the discussion

Astwood Dr Li s extensive investigations on corticotropin extend ing as they do over nearly ten years have stimulated numerous investi gations in this field His observations have been particularly significant in that they have substantiated the view that the hormone may not be a protein but rather a simpler molecule of quite reasonable stability and thus one which might reward attempts at isolation characteriza tion and eventual synthesis

It may be of interest to the members of the Conference to supple ment Dr Li s remarks by a brief summary of an investigation with which my associates and I have been concerned These studies com prising chemical investigations on corticotropin carried out in collab oration with Dr M S Raben and Dr R W Payne (13) and clinical observations made with the additional collaboration of Dr A P Cleroux and Dr I N Rosenberg (14 15) were made with the aims a) of developing efficient methods for the extraction of corticotropin in good yield in forms suitable for clinical use in order to conserve the limited supplies of pituitaries available and b) of purifying the active principle in order to aid in the elucidation of its chemical struc ture The clinical observations made it possible to evaluate the thera peutic effectiveness of various preparations as purification proceeded thus providing a check on the bioassay carried out by the method of Sayers Sayers and Woodbury (16)

As reported to a Macy Conference on the Adrenal Cortex held here eight years ago (17) acetone dried pig pituitary powder proved to be the most suitable source of the hormone More recent studies on the glacial acetic acid extraction method used then have shown that treat

that is material which was made into solution in 1 M HCl at room temperature?

Li Yes we did—the activity of the treated preparation has the same ACTH potency as the original untreated material If you let it stand longer the activity would be increased

Sayers *Apparently activation occurs?*

Li When a hormonal solution in 0 05 M HCl was put into a boiling water bath for thirty minutes no hydrolysis was observed but the ACTH activity appeared to increase three to five times

Sayers You claim there is activation?

Li Yes

White Have you examined that simple procedure for its efficacy in the removal of posterior pituitary principle? For example boiling for half an hour in 025 M HCl What is the result?

Li As far as the antidiuretic factor is concerned there is no change of its activity in 0 05 M HCl for thirty minutes heat treatment Dr W O Reinhardt and I have recently discovered that if you boil an ACTH solution in 0 5 M HCl antidiuretic contaminants will be de stroyed almost completely

Sayers Does it apply to both hog and sheep preparations?

Li We don t know

Sayers I am interested in getting rid of posterior pituitary activity from hog preparation We have not as yet boiled ACTH in 1 M acid However ACTH does not lose or gain activity when boiled for one hour in hydrochloric acid at pH 1

Crude extracts high molecular weight preparation and low molecular weight preparations have all been tested for stability at pH 1 at 100 C They neither gain nor lose activity

White What concentration of protein Dr Sayers?

Sayers Concentrations of 10 mcgm per ml to 1 mg per ml for the purified preparations and 2 mg per ml for the crude extracts of pituitary tissue

Li I think the difference might be in the conditions In very low concentrations you might not find similar activation In the high con centrations the rate of the hydrolysis might differ depending upon the concentration in the substrate

Thorn If this material is completely devoid of posterior pituitary activity i e oxytocic has it ever been tested on animals maintained on a constant water intake and preferably adrenalectomized for the antidiuretic activity?

Li We hope to find out in our future studies

Ralli We have noted the antidiuretic activity of various preparations

TABLE VIII

Adsorption of Crude Corticotropin on Powdered Cellulose from 0 1M Acetic Acid and Elution of the Active Fraction by 0 1 Hydrochloric Acid

Preparation	Dimensions of Column Di meter × He ght	Weight of Cellulose	Weight of Crude Extract	Amount Recovered In Acet c Acid	Amount Recovered In HCl El ate	Minimal Effective Dose
	m	gm	gm	c'	%	mcgm
1 25	3 3 × 32	75	1 5	67	8 7	0 1
2 9	7 5 × 19	225	4 0	82	8 5	0 025
2 19	7 5 × 21	250	5 0	82	9 0	0 05
2 24	7 5 × 21	250	5 0	76	7 9	0 05
2 25	3 3 × 22	50	1 0	75	9 7*	0 05
3 14	13 2 × 7	250	5 5	79	7 6	0 1
3 15	3 3 × 22	50	1 1†	69	5 1*	0 1
3 29	3 4 × 86	200	5 0	—	5 9	0 025
4 1	7 5 × 25	300	5 7	72	9 2	0 1‡
5 24	14 2 × 30	1 250	25 0	71	7 7	0 05‡
6 10	8 4 × 80	1 250	25 0	—	8 0	0 05

0 1M H SO used n t d f HCl

†C ude t act fi t t d 0 1M t c d w th 1/80 of its we ght of p ps n fo th rty min t

‡G ter than m nim l pons lower dos s not tested

Thorn I would like to ask Dr Sayers what the potency is of the original material from which La 1 A is obtained Do you know what the best assays were on that original batch of material?

Sayers I don t understand the point La 1 A is a solid For stand ardization purposes an unknown is compared in terms of a weighed amount of La 1 A

Thorn That is right but La 1 A was not the original substance obtained

Sayers I believe La 1 A refers to the fact it was a lactogenic hor mone preparation It was not anticipated that it would have marked ACTH activity

Long It is an impure standard?

Sayers Yes

White You can open one of the vials and see how much is in the vial and on the vial it states what the equivalent to La 1 A would be

Pincus We have opened them and have found various amounts We sometimes found 1 mg equivalent to 1 mg of La 1 A and in another

ment of the anterior lobe powder with a sixteen fold weight of the acid at 70° removes the activity quantitatively If to this extract is added a one half volume of acetone in the presence of 0 025 M NaCl a large precipitate forms which contains the thyrotropin gonadotropins and luteotropin contained in the extract Corticotropin is then removed from the solution by the addition of an equal volume of ether The resulting dry powder, constituting about 12 per cent of the weight of the starting material is about twice as active as the standard La 1 A and can be used directly in man without further purification This simple method requiring only twenty four hours to carry out thus gives a clinical product containing nearly all of the activity originally present Some 177 patients have been treated with this preparation

When used in man it turned out that 15 mg every eight hours proved to be effective as a therapeutic dose probably equivalent to 100 mg daily of La 1 A Extracts made from the pituitaries of other species which are of lower potency and even those made from the whole pituitary of the pig require further purification before use in man

A simple process has been worked out which provides a ten to fifteen fold purification without significant losses Advantage is taken of the property of the active principle to adsorb on cellulose from weak acid solution and to be eluted therefrom by stronger acid The crude extract in 0 1 M acetic acid is passed through a column containing a fifty fold weight of powdered cellulose After washing thoroughly with dilute acetic acid the active principle is removed with 0 1 M HCl The yield averages 7 per cent by weight of the crude extract and as this preparation assays twenty to thirty times La 1 A recovery of the active principle is nearly quantitative Clinical trials on 49 patients indicate that 1 mg every eight hours constitutes a full therapeutic dose roughly equivalent to 45 mg of the crude extract or to 100 mg of La 1 A

Ralli How long did you wash the column Dr Astwood?

Astwood The column is washed with 0 1 M acetic acid until no further material emerges

On the basis of the biuret or the optical density of 275 millimicra Table VIII shows that in eleven experiments the amount of the active fraction ranged from 5 1 per cent to 9 7 per cent and averaged about 7 per cent of the crude extract used Similar results were obtained whether small columns 3 3 by 22 cm containing 50 gm of cellulose were used for the fractionation of 1 gm of material or proportionately larger columns and 25 gm of the crude extract

We observed a much larger increase in amino nitrogen than Dr Li did

Li After pepsin?

Astwood Yes Whether the hormone is being hydrolyzed or not we cannot say from the data

Li Before pepsin is there precipitation with the trichloracetic acid?

Astwood The quantity precipitated by trichloracetic acid depended upon the concentration In the usual concentrations about half was precipitated All precipitations have tended to split the activity between the precipitate and the supernatant

In 0 1 M HCl by the successive addition of sodium chloride we removed precipitates in ten fractions every precipitate was active and the 5 M NaCl supernatant was still active From this sort of experiment we have assumed perhaps incorrectly that the active material has an extraordinary tendency to adsorb to solid phases

Li For example it is necessary to assay the trichloracetic acid extract repeatedly and then assay it again Have you done that?

Astwood No in the case of cellulose for example you can wash cellulose to which the material is adsorbed forever without removing the activity when the pH is above 2 5

Figure 6 shows comparative assays on the several preparations referred to The line marked 62 AA was taken from the data of Sayers Sayers and Woodbury our data were plotted and the regression lines drawn parallel to that of 62 AA We obtained variable results with La 1 A because of the fact we had two samples which seemed to us to differ by a factor of 2 In any event the crude extract that we had been using does appear to be about twice as active as La 1 A The hydrochloric acid eluate proved to be between ten and fifteen times as active as the crude extract and the materials further purified using picric acid or sulfosalicylic acid and countercurrent distribution were roughly one hundred times as active as La 1 A

Whether or not the hormone itself is a peptide is a question which has not yet been settled Mild treatment with pepsin alters the properties of the extract without affecting the potency It is not known whether the hormone itself is hydrolyzed under these conditions Prolonged treatment with pepsin destroys activity but this probably does not establish the peptide nature of the active principle in view of the possibility of side reactions It would appear that some novel method of fractionation must be devised before it will be possible to obtain the hormone in pure form

Li What happens if you take the two hundred fold active material of pepsin digestion? Did you destroy the activity or retain it?

bottle we found 5 mg equivalent to 1 mg of La 1 A

White That has not been our experience We have had material which on the basis of weight runs about thirty times as potent as La 1 A If you weigh the contents of a series of vials you can compare the weight in relation to what it is said to be in terms of La 1 A

Astwood To resume The preparation made by cellulose adsorption and elution is a suitable starting material for further purification One can neutralize the excess hydrochloric acid present with an exchange resin and freeze dry this preparation The potency can be doubled by any one of several procedures including retreatment with cellulose precipitation by picric acid and solvent partition The presence of appropriate concentrations of various strong organic acids such as tri chloracetic sulfosalicylic benzene sulfonic or picric acids permits the distribution of material between organic solvents and water Application of the Craig countercurrent distribution method to material purified with cellulose using various solvent systems has resulted in a further fourfold purification thus providing material approximately one hundred times as active as the standard La 1 A

This degree of purification is difficult to reconcile with Dr Li s concept that the hormone is a part of a protein molecule If these concentrates are one hundred times as active as the so called pure protein of 20 000 molecular weight the active component would have a molecular weight of less than 200

Thus far the purest preparations still appear to be mixtures and it thus has not been possible to determine the chemical nature of the active principle The material has been found to be stable in 0 1 M HCl for many months in the icebox and in confirmation of the work of Collip (18) and of Dr Li it is stable for several hours at 100 C in 0 1 M HCl but not in 1 M It appears to withstand treatment with mild alkali but the activity sometimes is unaccountably lost during manipulations at neutral pH These findings agree with those of Ghosh *et al* (19) The activity appears to be destroyed by oxidation and by certain reducing agents The active principle appears to have strong basic properties as indicated by altered solubility characteristics in the presence of organic acids and by the property of adsorbing on acidic solids The hormone appears to have a strong tendency also to adsorb to precipitates this property hampers purification and it may explain the diverse physical properties of various active fractions The purest preparations which we have examined react with both the ninhydrin and the biuret reagents and exhibit adsorption maxima at 275 millimicrons The ninhydrin reaction is greatly increased after strong acid hydrolysis indicating the presence of peptides in the extract

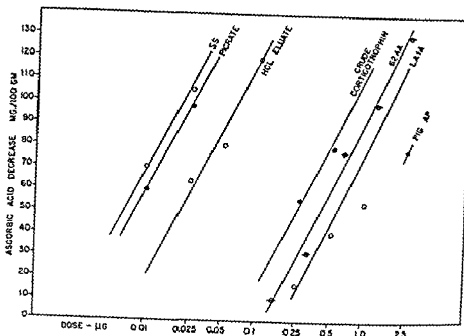


FIGURE 6 Assays of various preparations of corticotropin by the method of Sayers Sayers and Woodbury (17) From right to left the substances tested are acetone dried pig anterior pituitary powder the La 1 A standard preparation 62 AA plotted from the data of Sayers Sayers and Woodbury crude corticotropin made by the glacial acetic acid method material purified by adsorption on cellulose and elution with 0 1 M HCl and two preparations purified by the Craig countercurrent distribution method using 2 butanol and aqueous picric and sulfosalicylic acids respectively

Astwood We have not done that but we have treated material obtained by the cellulose method with pepsin Within the first hour or so there is a very marked increase in amino nitrogen without apparent loss of activity whereas when peptic digestion was continued overnight there was loss of activity We have not treated the more purified fractions with pepsin

Pincus How do you deduce this figure of 200 for molecular weight?

Astwood The molecular weight of the so called pure protein hormone is said to be 20 000 and if one purifies the material one hundred fold one would have to put the decimal point over two places to the left to give 200 as the maximal molecular weight of the presumed protein fragment Probably the most refined fractions are not pure so the molecular weight would have to be smaller still

My conclusion is I regret to say in the presence of many friends that the pure proteins of Sayers White and Long and of Li Simpson and Evans, appear from the present evidence to have been contaminated with the active principle

Sayers I would be willing to give the 20 000 molecular weight ACTH a well deserved burial at this point!

Li What is your estimate of the molecular weight of this active principle that you have?

Astwood I cannot tell because it is still a mixture I don t think any estimate of the average molecular weight of a mixture would mean anything Supposing the active principle had a molecular weight of 100 and is contaminated with protein of 100 000 molecular weight an average would be meaningless

Li It would give you some idea what the average is I am not trying to say your active material is 200 or 200 000 molecular weight but I think you can get an idea generally regarding the sphere of the molecular weight

Astwood We have not felt it worth while to try

Li You say when you put the active material in alkali it will withstand that treatment?

Astwood It will stand about 1 M ammonia

Li How about sodium hydroxide?

Astwood It loses its activity with strong base but in 0 1 M sodium carbonate overnight it retains activity

Pincus Have you assayed any preparations for posterior lobe hormone?

Astwood I am afraid I have not However the crude extract can be used in patients without side effects attributable to posterior lobe hormones

Thorn We can confirm the potency of the earlier extract which Dr Astwood prepared They were very potent on a milligram basis according to our clinical assays We do not however run any assay curves on these preparations using normal subjects

Pincus Did you not see any posterior lobelike side effects?

Thorn We did not with the early material but that depends to a great extent on whether one is looking particularly for this action Unless a patient is maintained on a constant water intake one might very well miss an antidiuretic effect since it is very transient

Ralli I wonder if I could chip in a few remarks on the posterior lobe and the nature of the crude extract because we have been using this material experimentally in rats not in patients and the Armour Company very kindly has provided us with a variety of fractions Some

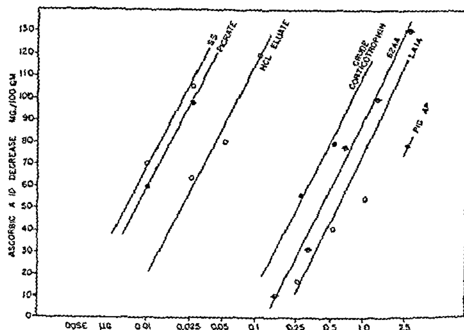


FIGURE 6 Assays of various preparations of corticotropin by the method of Sayers Sayers and Woodbury (17) From right to left the substances tested are acetone dried pig anterior pituitary powder the La 1 A standard preparation 62 AA plotted from the data of Sayers Sayers and Woodbury crude corticotropin made by the glacial acetic acid method material purified by adsorption on cellulose and elution with 0 1 M HCl and two preparations purified by the Craig countercurrent distribution method using 2 butanol and aqueous picric and sulfosalicylic acids respectively

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TABLE IX

Antidiuretic and Chloruretic Effect of ACTH

ACTH Lot No	*Dose* ACTH mg	Dose Equivalent in La 1 A *ACTH* Standard mg	Minimum Required for *Excretion of* 25% of Ingested Water	50% of Ingested Water	Chloride in First 25% of *Urine* Excreted mEq/min	Approximate Antidiuretic Potency in Milliunits of Pitressin/Rat
Control 0.01 M Acetic Acid			114	146	2.5	
71 2(50) C	1.0	0.2	216	not achieved	7.8	—
71 2(50) C	0.2	0.04	174	222	5.9	50
128 105 R	*2.0*	*3.2*	*168*	*223*	*9.3*	*40*
128 105 R	1.0	1.6	160	225	3.5	30
H 5409	1.0	0.9	225	not achieved	9.2	—
H 5409	0.2	0.18	195	not achieved	7.3	—
H 5409	*0.1*	*0.09*	*165*	*210*	*8.0*	*40*
V 16609	0.2	0.44	141	174	3.7	10
V 16709	0.2	0.25	129	159	1.2	5

significant chloruretic effect in the rats. An estimate of the antidiuretic potency of the various lots of ACTH tested is given in the last column of Table IX in which the potency is expressed in terms of milliunits of pitressin.

In view of the fact that sodium thioglycollate treated pitressin loses *its chloruretic effect in the rat* we *treated ACTH* with sodium thioglycollate and assayed this (Figure 7). Sodium thioglycollate treated ACTH retained its antidiuretic potency in the assay in the rat but lost its pressor and chloruretic effects.

The experiments indicate that considerable antidiuretic and chloruretic activity may be present in commercial ACTH the amounts varying in different preparations. The antidiuretic effect is undoubtedly due to contamination by the posterior pituitary.

The effect of treating ACTH with sodium thioglycollate was similar to the effect of this reagent on pitressin. In both instances assays in rats showed a loss of pressor and chloruretic *activities with only little* change in the antidiuretic activity.

Thorn Is it true Dr Ralli that the thioglycollate does not inactivate urinary antidiuretic principle?

Ralli Not in the rat but the antidiuretic effect is destroyed for the dog. We feel on the basis of our work with pitressin in rats (22) that there may be two antidiuretic fractions in pitressin. I wondered

are obviously crude and when I hear Dr Li and Dr Astwood speak I begin to realize that the preparations are cruder than even we had anticipated I don t know if others of you have found some of the commercial preparations toxic to rats

Long We have

Ralli We have been interested in studying the effect of ACTH on the white blood cell and eosinophil response in intact and adrenal ectomized rats Following the injection of ACTH some bizarre re actions occurred in the rats suggesting impurities in the hormone For this reason we did antidiuretic assays by the Burn method (20) Each rat was hydrated by gavage to 5 per cent of its body weight The sub stance to be assayed was injected intraperitoneally Rats in groups of four were placed in metabolism cages set in large glass funnels and urine was collected directly into graduated cylinders the volume ex creted being noted at fifteen minute intervals The experiments were continued until 50 per cent of the ingested water had been excreted or for two hundred and forty minutes if 50 per cent excretion was not achieved Chloride determinations were done on urine samples repre senting 25 per cent of the ingested water Five different lots of Armour ACTH were tested Lots 71 2 (50) C 128 105R and H5409 were assayed in duplicate at two or more dose levels Single assays were done on lots V 16609 and V 16709 The ACTH was dissolved in 0 01 M acetic acid (pH=3 4) and 1 ml of the solution was injected intraperitoneally The rate of urine excretion was compared with that of control rats given 1 ml of 0 01 M of acetic acid In addition one lot of ACTH (H5409) was treated with sodium thioglycollate accord ing to the procedure of Ames *et al* (21)

The results of the antidiuretic assays of the five different lots of ACTH are summarized in Table IX Dosage is given as milligram of ACTH administered to each rat and also as the amount of the Armour Standard La 1 A to which the ACTH dose was equivalent The latter is based on assays for ACTH potency supplied by the Armour Labora tories The antidiuretic potency is reported as the time required for the excretion of 25 per cent and of 50 per cent of the ingested water The chloride concentration determined on the urine sample represent ing 25 per cent of the ingested water is reported in microequivalents of chloride excreted per minute All the samples of ACTH tested contained significant amounts of antidiuretic material although the antidiuretic potency of the individual lots varied considerably as shown by the increased time required for the excretion of the ingested water With the exception of lot V 16709 all preparations of ACTH had a

been doing we have been trying to identify the antidiuretic material in urine We used sodium thioglycollate at the suggestion of Dr van Dyke because of its effect on destroying the antidiuretic activity of pitressin when assayed in the dog We found as did van Dyke (21) that thioglycollate does destroy the antidiuretic activity of pitressin when assayed in the dog but simultaneous assays of the same thiogly collate treated pitressin assayed in rats did not show a comparable decrease in antidiuretic activity The chloruretic effect however was decreased by thioglycollate

White Have you tried a thioglycollate treated preparation in the dog at a much higher dose level?

Ralli The pitressin activity is destroyed for the dog at fairly high concentrations of pitressin according to van Dyke (21) We did find in later experiments in the rat that if the amount of sodium thiogly collate was increased with respect to the concentration of pitressin the antidiuretic activity of the pitressin thioglycollate mixture was some what reduced We have not completed these experiments

White Was the relative sensitivity in the two species the same to the same dose of pitressin? Is this a response in sensitivity which you are altering? I am trying to propose an alternative explanation

Ralli There probably is a species difference but it may be tha the species difference is due to the presence of two different fractions

White I don t know I may point out however that the same prep aration of highly purified renin is diuretic in the rabbit and antidiuretic in the dog

Ralli As the dose of pitressin is increased in the rat there is an in crease in the antidiuretic response This was found by Jeffers (23) and confirmed by Stueck (24) The response to a given dose of pitressin is logarithmically related to the dose When the dose of pitressin is below 5 milliunits the rat assay is not as accurate as at the higher levels The accuracy of the assay can however be increased as shown by Gaunt (25) if the rats are triply hydrated before the in jection of pitressin

Thorn One has to be particular in this regard and I feel that Dr White has raised an important point If one assays posterior pituitary antidiuretic hormone in man that is a patient with diabetes insipidus in a dose sufficient to restore water balance to normal one will observe sodium chloride retention as a significant action of the hormone in contrast to the increased chloride excretion observed by Verney in his heart lung preparations

Conn I might add something to Dr Thorn s comment with which I agree I think everybody who has used Armour preparations of ACTH in normal individuals has been aware of pitressin like effects

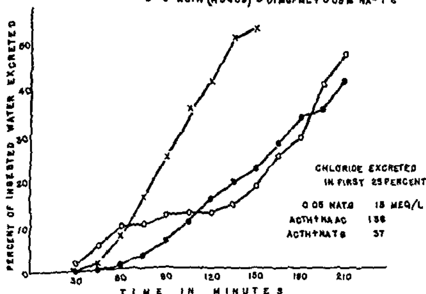


FIGURE 7 * Effect of sodium thioglycollate on ACTH Antidiuretic assays in rats of (x x) 1 *ml of* 0 05 M *sodium thioglycollate* (o o) 0 1 mg ACTH in 1 ml 0 05 M sodium acetate and (• •) 0 1 mg ACTH in 1 ml 0 05 M sodium thioglycollate

in listening to Dr Astwood and Dr Li if pitressin which has a fairly large molecular weight might not be broken down to smaller fraction as a result of treatment with thioglycollate

Li I would like to ask Dr Astwood once more about his methods Have you tried the same method in beef or sheep?

Astwood I am sorry to say we have not

Pincus You have always used anterior lobe from which the posterior lobe was dissected?

Astwood Almost always If the whole pituitary is used then there is enough posterior lobe hormone to give trouble in patients The crude extract is perfectly all right if made from the anterior lobes

Sayers I would like to assure Dr Astwood that his assay procedure is perfectly adequate It is necessary to use only a few rats when his chemical fractionations produce such marked increases in potency

Selye Is anything known about the mechanism by which sodium thioglycollate works?

Ralli Not that I know of Dr Selye In the studies that we have

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* Reprinted from *J Biol Chem* 187 719 (1950)

Ralli With the purest ACTH material that we were sent by Armour we obtained much less antidiuretic effect than we got from the cruder extracts This was with a purified beef ACTH

Thorn It is obvious that the original relatively crude ACTH preparations which we used initially had a rather marked posterior pituitary effect characterized by blanching of the skin intestinal cramps and water retention The more purified materials which we have been using recently have not exhibited this Dr Li s preparation interested me greatly because this is the first report of any material which still could be demonstrated to have ACTH activity with no antidiuretic activity whatsoever One can only establish the absence of antidiuretic activity in ACTH preparations in adrenalectomized animals or in patients with Addison s disease in order to eliminate water retention secondary to sodium and chloride retention following adrenal activation

Loeb In support of Dr Long one might mention the unmentionable desoxycorticosterone It is certain that with DCA administration in the Addisonian you may have water retention considerably in excess of sodium retention for a time as judged by actual gain in weight without any change whatsoever or even a fall in serum sodium concentration So there one has a situation of water retention out of proportion to sodium retention over a period of time in that the serum sodium concentration is not restored to normal That rectifies itself later on in most patients but as an immediate effect one sees water retention out of proportion to sodium retention

Ralli You don t get that in a matter of two or three hours do you?

Loeb In twenty four hours

Ralli If you were to hydrate a patient and give a reasonable dose of desoxycorticosterone and no salt was given just water would you get water retention?

Loeb I don t know

Thorn We have also reported that the level of serum sodium may drop in conjunction with sodium chloride excretion exhibited with desoxycorticosterone treatment when sodium chloride intake is minimal and the fluid intake is constant Because of the very close relationship between desoxycorticosterone cortisone and the posterior pituitary hormones I would not like to claim that this effect is mediated entirely by desoxycorticosterone but may possibly represent a posterior pituitary enhancing effect

Long I don t know that the experiment has been done but it seems to me that an adrenalectomized animal well hydrated and given desoxy corticosterone—taking that as a prototype of a salt retaining steroid—

We had the interesting experience of treating a patient who first had developed diabetes insipidus and then progressed on to destruction of the anterior lobe, at which time clinical evidence of diabetes insipidus had disappeared Then upon administration of ACTH the diabetes insipidus returned The latter could be controlled by the addition of pitressin The ACTH used is the same material which when given to a normal person suggested excess of pitressin like activity Yet the contaminating substance did not seem to take care of the pitressin deficiency found in that patient Perhaps it was a matter of relative amounts

Ralli When you are treating a human subject with a disease of the pituitary gland you really do not know the exact condition that you are treating You have to realize that the degree of damage to the pituitary varies and is something which one has difficulty in evaluating We have a patient with diabetes insipidus who has developed adrenal cortical insufficiency which has required continuous treatment with DCA and adrenal cortical extract We cannot define the degree or the type of damage and until anatomical studies can be made I doubt if we can do more than infer the degree of damage

Long May I ask for clarification? If one had an absolutely pure preparation of the adrenocorticotropic hormone and if one gave it to a well hydrated rat would not water retention occur?

Thorn You should not get water retention in excess of sodium retention with pure ACTH in amounts of ACTH which would induce a sodium retention One would expect a certain quantity of water retained in an obligatory fashion Our studies on commercially prepared ACTH often demonstrated a water retention in the first forty eight hours of treatment in considerable excess of sodium and chloride retention

Long Would you not get obligatory water retention?

Thorn Yes

Long I think you showed a good many years ago that after the injection of adrenal cortical extract there was both water and salt retention

Thorn That is true We never obtained retention of water in excess of normal electrolytes in the body With ACTH the picture may be entirely different One may observe the retention of 2 to 3 liters of water with only 40 to 50 mEq of sodium

Long The point Dr Ralli made was that after treating the extract with thioglycollate the chloruretic effect was no longer observed but water retention was still observed to occur Why then argue that you still have posterior lobe activity there When you had pure ACTH would you not expect the same thing?

Thorn I believe it is possible to clarify this point in relation to Dr Loeb s observation and Dr Astwood s Patients with Addison s disease have a high urinary antidiuretic assay and are unable to handle a water load with a normal diuresis If such patients are treated with desoxycorticosterone they are still unable to handle a water load ef ficiently and they continue to have a high antidiuretic assay If on the other hand these same patients are given cortisone supplementary treatment they no longer show the excessive antidiuretic effect and the antidiuretic assay in the urine may fall to normal levels The point I would like to stress is the fact that patients with Addison s disease treated with desoxycorticosterone will retain sodium and chlo ride as a result of this hormone therapy and will in addition have the excessive water retention dependent upon the elevated antidiuretic activity There is no evidence of inhibition of posterior pituitary activity with desoxycorticosterone treatment Now when one analyzes the effect of ACTH we cannot state precisely what hormones are elaborated It appears as though the predominant one is an 11 17 oxysteriod One usually observes an initial phase of salt and water retention following ACTH treatment after which there is a leveling off and in the later stages of treatment one may actually observe a spontaneous diuresis in a patient on ACTH or cortisome treatment One can always obtain a good sodium chloride diuresis but I do not know of any form of therapy which would give these patients a pure water diuresis other than stopping the ACTH

Bloch I would like to come back to the chemistry of ACTH and raise one point It seems to me that since ACTH is assayed *in vivo* it is impossible to say whether the active principle is a protein or peptide I think all the data which have been presented could very well be reconciled if you assumed that the smaller fragments are reconstituted to the hormone *in vivo* unless you find certain peptide fractions which do have a very much higher activity per unit weight than the protein If that were the case then one should perhaps find a condition where reconversion of the peptide to the protein cannot take place any more I wonder whether adrenal perfusion as used by Dr Pincus and Dr Hechter might not show differences betwen the peptide fraction and the protein Have you tried that?

Pincus We would like very much to try highly purified material as against the protein

Thorn Have there been no reports of successful studies utilizing the addition of purified ACTH to adrenal cortical slices as *in vitro* studies It would appear to me that one ought to observe profound changes in the metabolism of isolated adrenal cortex when ACTH is added to the substrate

would show a change in water balance quite soon after the injection

Thorn That is right But Dr Loeb s point whether you get excessive water retention or not, is related to some extent to the available sodium chloride in the diet A lot of salt in the diet tends to increase serum sodium concentration

Loeb In the presence of a hypoalbuminemia, Dr Thorn, one frequently sees considerable and rather rapid gains in weight without significant increase in serum sodium concentration even with a fairly liberal intake in sodium

Long We are getting perhaps a little away from ACTH and I would like to ask Dr Astwood whether with his new preparation he has observed any of the other previously reported effects of ACTH such as the increase in liver glycogen deposition in the normal animal

Astwood I am afraid we have not done this Dr Long Clinical observations on patients have confirmed the fact that all the metabolic effects heretofore described are obtainable with the purest preparations that we have used There is no qualitative difference that we can detect We do not have sufficiently accurate data to say whether there is a difference attributable to a lower content of posterior lobe hormone but the tendency to retain water and to develop edema is the same with the purified material as with crude extract

Long What I am obviously trying to determine is whether there are any differences in the biological activities of these different preparations

Astwood I think the similarity of the clinical response suggests that there is no evidence of a second active principle

Long So far as you know?

Astwood Cushing s syndrome can be produced with equal facility with crude or with purified preparations The various features of this syndrome encompass most of the physiological phenomena of which you speak

Pincus This is always with equivalent doses in terms of Sayers assays?

Astwood Yes

Dougherty I would like to ask Dr Astwood whether the amount of ACTH given for rheumatoid arthritis was 15 mg per dose or 15 mg per day

Astwood Fifteen milligrams of the crude extract every eight hours That would be 45 mg per day of the crude extract which would be equivalent to 100 mg of the La 1 A standard

Ralli If you have a patient on ACTH who has retained undue amounts of water can you produce diuresis by the use of a diuretic?

a reduction in apparent potency

Sayers We have some experimental data that may be of interest to Dr Pincus in connection with adrenal perfusion Mr Richards and I were interested in the stability of the adrenocorticotropic hormone in blood *in vitro* Our early studies indicated that ACTH was rather unstable in blood The possibility had to be considered that a proteolytic enzyme was contaminating our ACTH preparations and inactivating the incubated ACTH Our more recent studies do not rule that possibility in or out However as a precaution we now boil ACTH in 0 1 M HCl before incubating This treatment undoubtedly destroys all proteolytic enzyme activity ACTH prepared from rat pituitaries is boiled in acid and incubated with rat blood *in vitro* at 37° C for one hour During this period there is no loss of ACTH activity The rapid disappearance of injected ACTH from the blood was obviously not due to destruction of ACTH by blood

Rats were sacrificed five and fifteen minutes after the administration of the ACTH and the organs analyzed for ACTH content No activity was present in the liver We did find activity in the adrenals and a *surprisingly high amount of activity in the kidneys At fifteen minutes* there is just about as much activity in the kidney as there is at five minutes Apparently there is a very rapid concentration of the hormone in the kidney Kidney tissue has a concentration of hormone activity greater than that of adrenal tissue This is rather interesting in the light of the fact that the hormone is not detectable in the urine The hormone could be destroyed in the urine especially in the rat which excretes an alkaline urine However our studies indicate that destruction in alkaline rat urine is not rapid enough to account for the fact that there is no active hormone excreted

Thorn Did nephrectomy allow maintenance of a high level?

Sayers We have not performed experiments to test that possibility

Pincus The disappearance in blood that we observed was after one to four hours but I do agree with what Dr Astwood has said about the capacities of this material to be adsorbed It may be that all we were observing was essentially a binding by some blood constituent which did not release it fast enough at least for the Sayers assay to give us any indication because the Sayers assay is an hour s assay

Sayers You inject the blood itself into the rats?

Pincus Yes we found any attempt to extract the material that we could make resulted in loss of activity So we just had to use the whole blood or the whole plasma in our studies

Selye In reply to a question raised by Dr Long about the possible multiplicity of ACTH I think a few experiments from our laboratory

Bloch That brings up the whole problem of *in vitro* effects of hormones which are notoriously difficult to demonstrate

You might even expect that the peptide would be more active in the *in vitro* system than the protein hormone

Pincus We have some observations which are suggestive We have perfused adrenals with an Armour preparation of ACTH We found that with low concentrations over quite a range there was no difference in the quantitative production of corticosteroids in that range I reported that at our last meeting More recently we attempted to see what happened to our ACTH and we obtained a result which is in a way very discomforting When we incubate the ACTH with whole blood which is our medium a large proportion of the activity will disappear When we try to correlate that with our observation that after perfusion with ACTH the increased corticosteroid output is maintained for some time the only conclusion seems to be that a moiety of ACTH is fixed by the adrenal and continues to act over a period of time So the question is more complicated than you might think initially What we must and really have to know is how the active ACTH is fixed by adrenal tissue Until we can get that answer we are likely to be deceived for instance by quantitative studies with ACTH

Ingle I am interested in the possibility that the utilization of ACTH as affected by the mode of administration may be an important factor in determining the apparent quantitative activity of different preparations We have given ACTH by continuous subcutaneous injection to rats and have obtained much greater adrenal cortical hyperplasia and metabolic changes per unit of weight than can be obtained by intermittent injection I have heard of hydrolysates of ACTH highly potent by the Sayers ascorbic acid depletion test which failed to cause cortical hyperplasia when injected intermittently over a period of days Perhaps the peptides would appear fully active if given by continuous injection Dr F S Gordon of the University of Wisconsin (26) has given commercial preparations of ACTH by continuous intravenous and continuous subcutaneous injection in patients and was able to demonstrate a clinical response with smaller amounts than are required by intermittent injection

Thorn This is also true of posterior pituitary hormone The amount necessary to regulate water output if fractionated into one or two hour injections is very small

Astwood We can confirm that the purer materials appeared to be weak when assayed by the older methods involving twice daily injections for five days though they are highly potent by the Sayers method It was rather surprising to find therefore that administration of these purified materials to patients every eight hours did not reveal

If cortisone is given simultaneously with LAP it never causes pronounced adrenocortical atrophy since the LAP contains ACTH which stimulates the adrenal Hence the nephrotoxic action of LAP is always aggravated by cortisone

If STH is administered concurrently with small doses of cortisone the adrenocortical atrophy normally produced by the latter is inhibited Perhaps STH exerts a direct adrenotropic effect of its own but it is also possible that STH merely stimulates the discharge of endogenous ACTH by the animal s own pituitary and thus acts on the adrenal indirectly

STH (like LAP or DCA) appears to promote the formation of granuloma tissue around the joints in rats in which formalin had been injected into the joint region ACTH and cortisone have an exactly *opposite effect*

It seems to me therefore that STH is in some manner related either to the production or to the activity of mineralocorticoids We do not have any definite proof that it is directly corticotropin since its mineralo corticoid activity might be due to a peripheral sensitization of the tis sues to normally and independently produced mineralocorticoids

Pincus Do you get the result in adrenalectomized animals?

Selye We have not tried it

Li Nor in hypophysectomized?

Selye No I rather think in the hypophysectomized animals it will *not act as well Growth hormone is not adrenotropic according to* Dr Li

Long Isn t it? The California group reported some years ago that as the animal grew the adrenal grew as well

Selye Yes but only proportionately with the body weight increase I may say that LAP in hypophysectomized animals is very much less corticotropic (as judged by adrenal enlargement) than it is in the intact animal when given at the same dose level I was tempted to ascribe this to the nonspecific damaging action of LAP which being an impure and toxic preparation might produce ACTH discharge through the adaptation *syndrome mechanism This assumption is perhaps unjusti fied in* view of our recent findings with purified STH *It* is possible that the growth hormone itself has the ability to induce an ACTH discharge just as LH causes an FSH discharge and vice versa However this would lead us to too much speculation and meanwhile I merely wanted to report a few new facts

Sayers Three milligrams of growth hormone contain how much ACTH?

Li As far as we can detect the growth hormone preparation is practically free from other active contaminants For instance 1 mg did

might be of interest You may remember that some time ago we used lyophilized anterior pituitary (LAP) tissue and found that in suitably sensitized rats (unilateral nephrectomy and a high sodium intake) this substance imitated many of the actions which one can normally obtain with DCA or with Reichstein s compound S that is with mineralo corticoid substances

The question arose as to what principle in the LAP is responsible for these mineralocorticoid like actions of a hypophyseal prepara tion? Contrary to what happens when you give ACTH (the usual kind of corticotropin) animals treated with LAP had large thymus glands Even when the adrenal is enlarged to the same extent with LAP and with ACTH the former will tend to increase the weight of the thymus while the latter causes marked thymus atrophy If the rats are sensi tized to the mineralocorticoid actions they develop nephrosclerosis hypertension and myocarditis under the influence of LAP If ACTH is given under similar conditions and in amounts resulting in the same degree of adrenal enlargement it does not cause such lesions of the hyalinosis type Furthermore the spleen and the liver are greatly enlarged after LAP but not after ACTH treatment in the rat

More recently we have given ACTH and LAP simultaneously to rats sensitized for mineralocorticoid actions Here we found that ACTH actually aggravates the toxic actions of LAP mentioned above as far as the kidney and heart are concerned but it counteracts the thymus and spleen enlarging action of LAP This reminded us of previous experi ments in which DCA and cortisone were given simultaneously Here again only the nephrotoxic and cardiotoxic actions of the mineralo corticoid were augmented by the glucocorticoid compounds In most other respects these two types of steroids are antagonistic

Recently Dr Li was kind enough to send us some purified somato tropic hormone (STH) Since LAP is rich in somatotropin (or growth hormone) we wanted to examine whether the latter is responsible for the mineralocorticoid actions of LAP This appears to be the case In suitably sensitized rats we note that 3 mg of Dr Li s purified STH preparation given over a period of two to three weeks causes the development of the same organ changes as we have previously obtained with LAP that is a very large thymus and spleen with nephrosclerosis myocarditis hypertension and marked diuresis

We had previously noted that if LAP is given *simultaneously* with cortisone the nephrotoxic effect of the former is actually aggravated *Similarly* we now observe with the purified STH that concurrent administration of cortisone aggravates the renal effect but only if *cortisone* is given at a dose level which does not cause marked compen satory adrenal atrophy

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not cause depletion of adrenal ascorbic acid in the hypophysectomized male rats

I would like to come back to the question of ACTH destruction by animal tissues Some years ago we found that incubation of ACTH with homogenates and slices of rat kidney and adrenal and liver abolished the activity Recently Dr Geschwind fractionated the liver homogenate by differential centrifugation and discovered that the supernatant fraction is chiefly responsible for this inactivation

Experiments were carried out at 37°C we did not investigate the influence of temperature

Sayers I have wondered about the significance of experiments in which a tropin is incubated with slices or brei of a target organ or other glands Proteolytic enzymes destroy ACTH activity In the case of the adenohypophysis ACTH activity is not destroyed even at 37°C just as long as the integrity of the cells is maintained Crush the cells and ACTH activity disappears and in a gland in which the hormone is synthesized rapidly!

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an acceleration in the rate of discharge of ACTH from the adeno hypophysis is of relatively little significance when considered alone On the other hand it is significant that the disruption of a neural or neurovascular path, or the destruction of a nucleus of nerve cell bodies, prevents or inhibits the accelerated rate of discharge of ACTH which normally accompanies the application of stress to the organism

THE INDICES OF ADENOHYPOPHYSEAL ADRENO CORTICOTROPIC ACTIVITY

Evaluation of pituitary adrenocorticotropic activity is no better than the index employed to assess such activity Time does not permit de tailed analysis of the various methods employed to determine the rate of discharge of ACTH from the adenohypophysis the subject has been *previously reviewed* (1) *The experimental studies on regulation* which will be considered in the present report have with few excep tions employed one or more of the following indices adrenal weight adrenal ascorbic acid adrenal cholesterol circulating lymphocytes cir culating eosinophils The specific action of ACTH upon the adrenal cortex is manifested by an increase in weight of and a decrease in concentration of cholesterol and ascorbic acid in the gland These three phenomena are excellent indices of the rate of discharge of ACTH from the adenohypophysis Adrenal weight is of particular value in experiments of relatively long duration On the other hand adrenal ascorbic acid and cholesterol are admirably suited to experi ments of short duration

Increased rate of discharge of ACTH stimulates the adrenal cortex to increased rate of secretion of cortical hormone a substance which induces lymphocytopenia and eosinopenia Lymphocytopenia has been particularly valuable for the assessment of ACTH discharge in rodents In man the change in the number of circulating eosinophils is the best index of adrenocortical activity available However caution must be exercised in the interpretation of eosinophil changes particularly when the organism is subjected to a severe degree of stress It has for ex ample been demonstrated that large doses of epinephrine induce an eosinopenia in patients with Addison s disease (2)

METABOLITES

The adrenal cortex influences the concentration of a number of metabolites in the body fluids The adenohypophysis in turn may use the concentration of one of these substances as an index of the require

REGULATION OF PITUITARY ADRENOCORTICOTROPIC ACTIVITY*

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THE NEEDS of the animal for cortical hormone vary widely depending upon the activity and the nature of the environment of the organism. Stress induces prompt and dramatic changes in the histology and chemistry of the adrenal cortex indicative of enhanced secretory activity. That this enhanced secretory activity is in response to an increased need for cortical hormone is demonstrated by the fact that the requirement of the adrenalectomized animal or the patient with Addison's disease for cortical hormone increases markedly during periods of stress. The pituitary adrenocortical system is a dynamic system designed to meet the changing requirements of the organism for cortical hormone with the adrenal cortex the manufacturing plant and the adenohypophysis the director of production. The adenohypophysis through the mediation of the adrenocorticotropic hormone (ACTH) determines the secretory activity of the adrenal cortex. This afternoon I am going to discuss the mechanism or mechanisms which are concerned with the regulation of the rate of discharge of ACTH from the adenohypophysis. How does this gland interpret the needs of the organism for cortical hormone?

THE RESPONSE OF THE PITUITARY ADRENOCORTICAL SYSTEM TO THE GREAT VARIETY OF NONSPECIFIC STRESSES

Before considering theories of pituitary regulation it should be emphasized that a most characteristic feature of the adrenal cortex is its responsiveness to a great variety of nonspecific stresses. A relatively short period of apprenticeship in the field of adrenocortical physiology cools the ardor with which the novice attaches meaning to the discovery that a drug, hormone or environmental change stimulates the pituitary adrenocortical system. The demonstration that a substance induces

* The experimental work conducted in the author's laboratory was supported by grants from the American Cancer Society recommended by the Committee on Growth.

early decrease in the concentration of adrenal cholesterol which otherwise follows such exposure (24) The lymphocytopenic action of epinephrine can be inhibited by ACE (25) These numerous confirmatory observations in which a number of different stresses and indices of adrenocortical activity are employed strongly suggest that the great variety of nonspecific stresses stimulates the adrenal cortex to activity by increasing the requirement of the organism for cortical hormone Administration of cortical steroid obviates the necessity for the adrenal cortex to increase its secretory activity in order to meet the increased demands for hormone induced by stress

Since the tropic action of ACTH on the adrenals of the hypophysectomized animal as measured by increase in gland weight (5 6) and ascorbic acid depletion (21) is not influenced by administration of ACE it is reasonable to assume that cortical hormone acts to inhibit release of ACTH from the pituitary rather than to interfere with the action of the tropin on the adrenal cortex itself

Discharge of ACTH in response to moderate stress may be completely or partially blocked depending upon the dose of cortical steroid administered Futhermore with increasing intensity of stress the amount of cortical steroid required to suppress pituitary adrenocorticotropic activity becomes correspondingly greater (21 26) These quantitative relationships may be adequately explained if it is assumed that the rate of discharge of ACTH from the adenohypophysis fluctuates in accordance with the varying requirements of the organism for cortical hormone

Pretreatment with cortical hormone fails to block accelerated discharge of ACTH when the animal is exposed to severe stress Pretreatment with relatively large doses of cortical steroid partially but not completely blocks the reduction in adrenal ascorbic acid which normally occurs when rats are given large doses of histamine (21) Administration of ACE prevents the decrease in concentration of adrenal cholesterol which occurs in the first few hours after radiation but fails to prevent the late adrenal changes (24) Depletion of cholesterol in the adrenals of rats infected with *B tularense* is uninfluenced by large doses of ACE given at frequent intervals (27) Adrenal hypertrophy does not occur in the fasted rat given DCA (28) but neither DCA (28) nor ACE (29 30) affects the hypertrophy of fasting in the guinea pig Moya and Selye (31) and Gershberg *et al* (4) fail to confirm Sayers and Sayers (21) that DCA prevents the depletion of adrenal ascorbic acid which normally follows the application of stress stimuli The following explanations of these experimental facts may be considered First it is entirely possible that in the experiments where complete inhibition of pituitary adrenocorticotropic activity is not ob

ment of the organism for cortical hormone The studies are far too limited in scope to allow even tentative conclusions to be reached regarding this very important phase of the subject

Ingle and Kendall (3) were unable to influence the adrenal weight changes which accompany stress in rats by altering the intake of sodium or potassium Administration of glucose prior to exposure to cold does not inhibit the increased rate of discharge of ACTH which normally follows such exposure (4) On the other hand, glucose as well as adrenocortical extract will inhibit the adrenocortical stimulating effect of insulin (4) However adrenocortical extract in contrast to glucose exerts a blocking action on pituitary activity without preventing the hypoglycemia

CORTICAL HORMONE

A considerable body of indirect evidence can be marshalled in support of the concept that the titer of cortical hormone in the body fluids regulates the rate of discharge of ACTH from the adenohypophysis Adrenal atrophy follows the chronic administration of ACE (5 6) or DCT (7 8 9 10 11) DCA inhibits the hypertrophy of the adrenals which normally follows the application of a variety of nonspecific damaging agents (12) exercise (12 13) thyroxine (14) estrogen (15) or electroshock induced convulsions (16) The adrenal hypertrophy which follows thyroxine (17) administration or exposure to low atmospheric pressure (18) is inhibited by treatment with ACE (17) In man withdrawal of DCA is followed by the metabolic changes characteristic of Addison s disease (19) These chronic experiments may be interpreted to mean that cortical steroid administration inhibits pituitary adrenocorticotropic activity under these conditions

Ingle (20) was able to show that the hypertrophy of the adrenal cortex of the rat which normally occurs after twelve hours of forced exercise does not occur when animals are treated with ACE throughout the period of exercise Sayers and Sayers (21) have demonstrated that the drop in adrenal ascorbic acid which takes place one hour after the application of cold or of heat or after the injection of typhoid toxin epinephrine or histamine can be prevented by pretreatment of the animal with ACE or crystalline cortical steroids ACE has been shown by Long and co workers to inhibit the depletion of adrenal ascorbic acid that normally occurs after exposure to cold or after unilateral adrenalectomy (22) or administration of epinephrine (23) ACE administered just prior to exposure to radiation will prevent the

servation that epinephrine induces a lymphocytopenia in the hypophysectomized rat (33) and an eosinopenia in the hypophysectomized mouse (34) On the other hand ACTH but not epinephrine stimulates the production of chemocorticoids by the isolated perfused adrenal gland (35)

Sayers and Sayers (26) have shown and Long (23) has confirmed that the reduction in adrenal ascorbic acid produced by epinephrine can be prevented by administration of cortical steroid The lymphocytopenic action of epinephrine is prevented by cortical steroid treatment (25) These observations strongly suggest that epinephrine at least in small doses, acts like other nonspecific stresses and does not have a direct action upon the pituitary

Neither Dibenamine an agent which blocks the excitatory effects of both sympathin and epinephrine (36) nor tetraethylammonium bromide a substance which produces autonomic ganglionic blockade (37) influences the reduction in adrenal ascorbic acid which takes place in the rat in response to acute stress (38) The lymphocytopenia of tourniquet shock is of greater magnitude in rats treated with Dibenamine than in animals not treated with the adrenergic blocking agent (39) These observations do not lend support to a direct action of epinephrine on the adenohypophysis or associated structures however, negative experiments of this nature are not conclusive since it is possible that the action of epinephrine on pituitary effector cells is not influenced by the autonomic blocking agents employed

The completely sympathectomized animal is an important experimental tool which has not yet been fully exploited in the elucidation of the role of the sympathoadrenal system in the regulation of pituitary adrenocortical activity It would be of great interest to know the comparative responses of the adrenal cortices of intact and sympathectomized animals to a variety of both acute and chronic types of stress The completely sympathectomized dog shows a normal eosinopenic response to the injection of formaldehyde (40) Cannon (41) has demonstrated that the sympathectomized cat is hypersensitive to various environmental exigencies However the degree of hypersensitivity does not approach that of the adrenalectomized animal The completely sympathectomized dog is hypersensitive to insulin but resists heat cold and anoxia almost as well as do intact control animals (42) Furthermore the sympathectomized dog is capable of maintaining normal blood sugar levels during exercise (43) Homeostasis is not significantly threatened by total sympathectomy in man (44) These studies suggest that acceleration of adrenocortical activity in response to acute stress is not dependent upon the activity of the sympathoadrenal system neither does it appear that epinephrine is essential for

tained insufficient quantities of cortical steroids are employed rate of utilization of cortical hormone appears to be exceedingly great during severe stress Second it may well be that the cortical hormone titer mechanism is the only mechanism at work in mild to moderate degrees of stress whereas in more severe degrees of stress other mechanisms play a complementary role to accelerate the rate of discharge of ACTH Third, in severe stress with accompanying cardiovascular collapse anoxia or accumulated toxins may act directly upon the cells of the adenohypophysis to increase their permeability to ACTH

The titer of cortical hormones in the body fluids appears to play a major role in the regulation of pituitary adrenocorticotropic activity The exact nature of the process by which the concentration of cortical hormone in the blood influences the rate of discharge of ACTH from the adenohypophysis is unknown it is here that the peripheral humoral concept is particularly vague The concept emphasizes the determining role of the peripheral tissues in regulating pituitary adrenocorticotropic activity by dictating the rate of utilization of cortical hormone On the other hand it minimizes the role of central mechanisms which induce ACTH discharge without regard to tissue needs for cortical hormone

EPINEPHRINE AND OTHER AUTONOMIC DRUGS

The close anatomical approximation of the adrenal medulla and cortex together with the fact that they both play important roles in homeostasis naturally leads to some speculation regarding a possible integrative functional relation between the sympathoadrenal and pituitary adrenocortical systems

Epinephrine may act by any one or a combination of the following mechanisms to induce discharge of ACTH from the adenohypophysis a) it may act centrally on effector cells in the adenohypophysis or in the hypothalamus (hypothalamic stimulation would in turn activate the adenohypophysis) b) it may act like other nonspecific agents and stresses to increase tissue utilization of cortical hormone with a consequent lowering of venous titer of the hormone c) it may be the denominator common to all types of stress and the specific agent which promotes utilization of cortical hormone by the tissues

Vogt (52) from studies on the biocorticoid (cold protection test) content of adrenal vein blood reached the conclusion that epinephrine has a direct stimulatory influence upon the adrenal cortex However her experiments do not rule out the possibility that epinephrine acts via the adenohypophysis to bring about a discharge of ACTH Necessary but not sufficient evidence for the direct action thesis is the ob

from adenohypophyseal tissue by direct application of epinephrine is indeed an important contribution to the problem of pituitary regulation However it must be demonstrated that epinephrine and other sympathomimetic agents are relatively specific in their action on the pituitary transplant before it can be concluded that epinephrine plays a *special* role in the regulation of pituitary adrenocorticotropic activity For example it would be of interest to know if the direct application of *histamine or a cholinergic drug induces release of ACTH* from a transplant

Certain experiments are difficult to reconcile with the direct action of epinephrine thesis As pointed out above adrenal denervation in the rabbit does not inhibit the lymphocytopenia which follows painful stimulation of the subcutaneous tissues (48) and complete sympathectomy in the dog does not interfere with the eosinopenic response to formaldehyde injection (40) Finally it is claimed by Hume and Wittenstein (51) *that certain discrete lesions in the hypothalamus* block the normal eosinopenic response to epinephrine in animals with an intact adenohypophysis

HYPOTHALAMUS

Section of the infundibulum does not interfere with the response of the pituitary to acute stress in either the rat (52 53) or the dog (40) nor does it interfere with the response to chronic exposure to cold in the rat (54) These experiments which demonstrate that the adenohypophysis is not dependent upon *direct neural* connections with the hypothalamus are in keeping with the anatomical fact that few or no nerve fibers pass from the hypothalamus into the *pars distalis* However the experiments do not rule out the possibility that a vascular connection the so called hypophyseal portal system plays a role in the regulation of adrenocorticotropic activity Of interest in this connection is the demonstration (55 56) that the hypophyseal portal system regenerates after stalk section Furthermore de Groot and Harris (48) and Hume and Wittenstein (51) have *presented evidence* which suggests that the hypothalamic centers have a regulatory influence over pituitary *adrenocorticotropic* activity According to Hume and Wittenstein (51) lesions in the supraoptic nuclei of the dog did not alter the eosinopenic response to stress On the other hand paramedian lesions in the anterior hypothalamus and *at the juncture of* the middle and posterior hypothalamus abolished the usual eosinopenic *response of the dog to epinephrine* and to insulin The response to operative trauma was inhibited but not abolished Despite the indication of inertia of the pituitary adrenocortical system on the basis of

the action of adrenocortical hormone on effector cells for the addition of epinephrine to an infusion of ACE does not improve the muscular work performance of adrenalectomized rats over that of similar animals given ACE alone (45) Whereas the sympathoadrenal system is essential neither for the functional activity of the pituitary adrenocortical system nor for the response of effector cells to cortical hormone the integrity of the pituitary adrenocortical system appears to have a considerable influence upon the response of certain effector cells to epinephrine For example the pressor response to sympathetic stimulation is diminished by adrenalectomy and restored to normal by cortical steroid replacement therapy (46)

Gellhorn and Frank (47) claim that hemorrhage and subconvulsive electroshock cause a lymphopenia in normal but not in adrenodemedullated rats However de Groot and Harris (48) have shown that adrenal denervation in the rabbit does not interfere with the lymphocytopenia induced by pain It is recognized that the discharge of epinephrine contributes to increased adrenocorticotropic activity of the adenohypophysis in numerous situations of acute stress The common association of epinephrine discharge and increased secretory activity of the adrenal cortex however does not help answer the important question as to whether the action of epinephrine is a necessary and essential link in the series of events which lead to increased secretion of cortical hormone during stress or whether epinephrine acts as do other nonspecific stresses to increase the needs of tissues for cortical hormone

McDermott *et al* (49) have demonstrated that a transplant of the adenohypophysis will respond to the direct application of epinephrine with release of ACTH They interpret their findings together with those of Ingle *et al* (5) Sayers and Sayers (26) Cheng and Sayers (50) and Recant *et al* (40) to mean that there is a dual mechanism controlling the release of ACTH one phase of which depends on the activation of the sympathetic nervous system and the other on the utilization of adrenal cortical hormones (ACH) in the body Long and associates (22 23 49) have pointed out that both the sympatho adrenal and pituitary adrenocortical systems are stimulated to increased activity by a great variety of stressful conditions and they are inclined to the view that epinephrine is not in the usual sense a nonspecific agent They consider that the stimulation of the elements of the autonomic nervous system with concomitant release of epinephrine that occurs under a variety of conditions appears to be a major factor in the activation of the adrenotropic secretion from the anterior lobe From the adrenal medullae the epinephrine appears to be carried to the anterior lobe where it acts directly upon secretory cells to stimulate the release of adrenocorticotropic hormone The release of ACTH

and associates (59) have found that spinal anesthesia in man inhibits the eosinopenia which follows operative trauma According to Sayers and Sayers (26) barbiturate acts specifically in cold to prevent shivering an activity of skeletal muscle which is associated with increased rate of utilization of cortical hormone The results of additional experiments designed to evaluate the influence of barbiturate anesthesia upon the response of the pituitary adrenocortical system to a variety of stresses are awaited with interest The evidence at present available indicates that barbiturate inhibits the response of the pituitary to only one or at most a few specific types of stress it does not support the thesis that the hypothalamus is an essential element in the regulation of pituitary ACTH activity

THE STATUS OF THE PROBLEM

The titer of cortical hormone in the body fluids influences the rate of discharge of ACTH from the adenohypophysis The exact mechanism by which the cortical hormone inhibits the adenohypophysis is unknown The action may be direct or it may be mediated by a metabolite whose concentration in the blood is determined by cortical hormone activity In severe stress anoxia or toxins may act directly on the adenohypophysis to induce discharge of ACTH

The fact that adenohypophyseal transplants discharge ACTH in response to stress indicates that direct neural or neurovascular connections are not essential elements in the regulatory scheme However the experiments do not rule out the possibility that the hypothalamus has a modifying influence upon pituitary adrenocorticotropic activity There is experimental evidence which suggests that certain lesions in the hypothalamus interfere with the normal response of the adrenal cortex to stress as measured by eosinopenia and lymphocytopenia

The common association of sympathoadrenal and pituitary adrenocortical activity in response to stress is circumstantial evidence in favor of the view that epinephrine plays a special role in the discharge of ACTH However studies from various laboratories which involve completely sympathectomized adrenal demedulated and adrenal denervated animals appear to be incongruous The direct action of epinephrine to induce discharge of ACTH from an adenohypophyseal transplant has important implications It would be premature to conclude from this experiment that epinephrine has a *special* regulatory role until it is demonstrated that the action of epinephrine on the transplant is not shared by other vasoconstrictor agents

A neurohumor of considerable practical importance would be one which acted directly on the adenohypophysis to accelerate the rate of

the eosinophil test the dogs were not hypersensitive to insulin Lateral or unilateral median hypothalamic lesions had no influence upon the eosinopenic response to stress in the dog

According to de Groot and Harris (18), stimulation of the posterior region of the *tuber cinereum* or mammillary body but not other regions of the hypothalamus or the hypophysis induced a lymphocytopenia in the rabbit Lesions in the *zona tuberalis* and in the posterior region of the *tuber cinereum* or in the mammillary body abolished or diminished the lymphocytopenia of stress in the rabbit Similar lesions in the posterior part of the *pars distalis*, *pars intermedia* or in the infundibulum do not influence the response to stress

Long and associates (22, 23 49) have demonstrated that diencephalic lesions (exact *locus* not given) in the rat interfere with the early (one hour after application of stress) eosinopenic response of the rat to a number of stimuli However, Long does not interpret these results to mean that the hypothalamus secretes a neurohumor which acts upon the hypothalamus as do Hume and Wittenstein (51) and de Groot and Harris (48) Since section of the spinal cord below as well as above the site of a painful stimulus abolishes the eosinopenic response to stress Long considers the hypothalamus to be the mediator of epinephrine release from the medulla Stress stimuli act through afferent nerves to stimulate the hypothalamus which in turn brings about the discharge of epinephrine via efferents to the adrenal medulla Furthermore Long *et al* (22 23 49) have demonstrated that diencephalic lesions do not inhibit the second phase of the discharge of ACTH which is considered to be due to an increased rate of utilization of cortical hormone

Neither direct neural or neurovascular connections with the hypothalamus are essential for the discharge of ACTH from the adenohypophysis Transplants of the adenohypophysis in the anterior chamber of the eye will discharge ACTH in response to stress (53 49 57) The transplant experiments do not rule out the possibility that the hypothalamus has a modifying influence upon pituitary adrenocorticotropic activity

Long and associates (22 23 49) explain the results of Recant *et al* (40) who found that sodium pentobarbital acts to stabilize the eosinophil count and those of Sayers and Sayers (26) who noted that barbiturate anesthesia prevents the adrenal cortical response to cold, as a selective action of barbiturates upon the hypothalamus However it has been demonstrated that barbiturate anesthesia will inhibit the response to cold (26 58) but not to epinephrine (26) histamine (26) hemorrhage (58) or heat (58) Laparotomy induces an eosinopenia in rats anesthetized with sodium pentobarbital (49) although Roche

ness in blocking the discharge of ACTH which accompanies stress Sayers and Sayers claim that DCA can block the increased rate of ACTH discharge which normally follows administration of small doses of histamine However Moya and Selye and Dr Long s group cannot confirm this experimental work All of the studies were done on rats I have up to now said nothing about dosage This is an important aspect of the problem but unfortunately there is not enough time to go into this thoroughly In Dr Selye s experiments with DCA he used a very large dose what amounted to an anesthetic dose In Dr Long s experiments a dose of 4 mg per rat was employed which is a relatively large dose of the steroid

Selye I would like to say in support of Sayers and Sayers that we did not fail to confirm them It is only that we were unable to inhibit *completely* the discharge of ACTH under stress either with DCA or with cortisone if the stress was of sufficient severity

That corticoids (as well as testoids) can diminish ACTH secretion during the alarm reaction is a fact which we have studied in some detail and have been able to demonstrate with a large number of steroids ever since our earliest publications on the adaptation syndrome The important point however is that there must be a mechanism other than the level of circulating corticoids which regulates ACTH discharge during stress Otherwise it would be impossible to understand how such an ACTH discharge could be effected in the presence of overwhelmingly large amounts of exogenous corticoids (60)

Sayers You do find partial inhibition occurs The experiments of Long indicate that glucose has very little influence upon the rate of discharge of ACTH He has shown that adrenocortical extract will block the effect of insulin in bringing about discharge of ACTH without preventing the hypoglycemia produced by insulin Glucose pretreatment had no effect upon the increased rate of discharge of ACTH which follows cold exposure The administration of cortical extract does not inhibit the action of ACTH on the cortex itself Dr Ingle has shown that adrenocortical extract has no effect on the action of the adrenocorticotropic hormone in increasing the weight of the glands in hypophysectomized rats In my own laboratory we have shown that adrenocortical extract has no effect on the action of ACTH in depleting adrenal ascorbic acid I am not entirely satisfied with these experiments and I would like to extend them with more quantitative studies I feel that one cannot definitely exclude the possibility that the adrenocortical steroids influence the action of the adrenocorticotropic hormone upon the adrenal cortex itself However I am quite sure that if they do have an influence it is a minor one

Thorn It is not a minor one One point we ought to keep in mind

discharge of ACTH, without regard to the concentration of cortical hormone in the body fluids Such a substance would substitute for ACTH and cortisone in the treatment of disease!

DISCUSSION

Dougherty May I insert a point here? I wonder if one of the problems which is concerned here is not the use of the term non specific It seems to me that as long as we use the term nonspecific stress it is very likely we may never delineate the basic problems clearly because they are too general The word nonspecific actually refers to a response common to many different stress stimuli rather than to the stimuli themselves So much emphasis is placed on the enhancement of ACTH and adrenal cortical secretion that it is frequently forgotten that among many stress stimuli some may induce specific responses as well as nonspecific responses other than those involving adrenal cortical secretion Accordingly stress could be defined as a stimulus which increases the secretion of adrenal cortical hormones The crucial questions which concern the function of the secretions of the adrenal cortex are then related to an analysis of the ways and mechanisms of action by which the response to stress modifies the other nonspecific and specific reactions to a stimulus

Sayers As far as the regulatory mechanism of pituitary activity is concerned nonspecific is a useful term Of course each stress has its own characteristic mode of action on other organs and systems In that sense it is specific I use the term nonspecific for lack of a better one

Long I wonder if you would state your views and perhaps outline what you consider to be the most salient points regarding the problems involved in the regulation of the secretion of ACTH

Sayers I have stated my views on the subject of the regulation of the pituitary adrenocorticotropic activity on numerous occasions I believe that it would be appropriate for the members of this group to analyze critically the various experimental studies pertinent to this subject It is generally agreed that histamine epinephrine atropine acetylcholine cold heat and hundreds of other agents or environmental changes will induce an accelerated rate of discharge of ACTH The discovery of additional pituitary stimulants adds little to our understanding of the mechanism involved Of course you may take the view that each stress stimulus acts in its own peculiar way to excite the pituitary to discharge ACTH That to me is a most confusing outlook With regard to the action of DCA there is disagreement as to its effective

ness in blocking the discharge of ACTH which accompanies stress *Sayers and Sayers claim that DCA can block the increased rate of* ACTH discharge which normally follows administration of small doses of histamine However Moya and Selye and Dr Long s group cannot confirm this experimental work All of the studies were done on rats I have up to now said nothing about dosage This is an important aspect of the problem but unfortunately there is not enough time to go into this thoroughly In Dr Selye s experiments with DCA he used a very large dose what amounted to an anesthetic dose In Dr Long s experiments a dose of 4 mg per rat was employed which is a relatively large dose of the steroid

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is that the rapid absorption and transient action of adrenal cortical extract by injection is in distinct contrast to the prolonged more effective action of cortisone acetate Experiments carried out with the use of aqueous adrenal extract unless the material is given fractionally every two or three hours may provide results entirely different from those observed by inducing stimulation of the gland with ACTH or substitution therapy with long acting cortisone

Sayers We have definitely established the fact that cortisone administered for a long period of time produces adrenal atrophy and the gland is no longer as responsive to ACTH as is a normal gland I believe you have shown that this is also true in man However these experiments have no bearing on the problem at hand which is concerned with the blocking action of injected steroids on a gland which is *not* atrophied Continued cortisone administration will induce atrophy of the gland to almost the same extent as does hypophysectomy Glands atrophied by cortisone or hypophysectomy are less responsive than normal to ACTH

Li In the normal animal, not the hypophysectomized animal?

Thorn In the normal animal

Ingle The prolonged administration of large amounts of ACE to normal rats causes compensatory atrophy of the adrenal cortices which can be prevented by ACTH When the adrenal cortices of the hypophysectomized rat are maintained at a normal size with ACTH the administration of large doses of ACE fails to cause compensatory atrophy (61) We now feel as does Dr Sayers that this type of information is not entirely satisfactory In addition to adrenal weight data careful histochemical studies are needed The observations of Dr Selye and his associates seem to make necessary the conclusion that the structure of the adrenal cortex and perhaps its secretory activity can be modified by conditioning factors whose effects are not mediated by ACTH

Thorn In order to make my point clear I think this example may be helpful A dose of 12 5 mg of cortisone twice daily will cause a marked involution of the adrenal in man as measured by the reduction in urinary 17 ketosteroid excretion The type of effect I have referred to is that induced by 100 to 200 mg of cortisone a level of hormone which I am sure has never been approached with adrenal extract experiments One would not expect that a physiological inhibition of ACTH would after a few days give an adrenal which would not respond to large doses of ACTH This failure to respond is highly suggestive that there may be a blocking action at the level of the adrenal cortex as well as an inhibition of ACTH as a cause for the marked

atrophy of the adrenal cortex which we observe in patients on long continued cortisone therapy

Conn Don t you think that may be related to the decrease in thyrotropin produced by large doses of cortisone the so called corticogenic hypothyroidism?

Thorn That is one possibility The point I am making is that the unresponsiveness of the adrenal may be due to a metabolic failure of a mechanism other than ACTH

Selye Did I understand you to say that in the hypophysectomized animal ACE fails to inhibit the corticotropic effect of ACTH?

Ingle That is true as far as the weight of the whole adrenal gland is concerned

Conn But the relative doses are different

Thorn The low dose causes a marked effect on ACTH secretion It takes a very large dose I think to get in the range of depression of other activity

Sayers Doses of cortical hormone in the physiological range did not interfere with the action of injected ACTH on the adrenal of the hypophysectomized rat Additional experimental studies are in order It would be foolhardy to say that cortical hormone has no influence whatsoever on the action of ACTH on the adrenal

Dr Long and his associates have studied the eosinopenic response to stress of adrenal demedullated rats rats with lesions in the diencephalon and cord sectioned rats They reach the conclusion that the modified response induced by these operative procedures may be explained by the failure of the animals to discharge epinephrine from the adrenal medulla which has a direct stimulatory action upon the adenohypophysis These conclusions are at variance with the results of Hume and of Harris Hume claims that epinephrine fails to induce an eosinopenia in dogs with paramedian lesions in the anterior hypothalamus Denervation of the adrenals of the rabbit fails to influence the lymphopenia which follows faradic stimulation of subcutaneous tissue in the rabbit

Pincus Isn t there some evidence that the epinephrine alone might cause eosinopenia in hypophysectomized rats?

Long Not in our experience

Thorn Nor in Hume s dog experiments However it may be a question of dose I think one always has to consider seriously the dose level when discussing this particular point

Long Probably what Dr Pincus is referring to is the paper from the Wisconsin laboratory where using the hypophysectomized mice a fairly large dose of epinephrine was followed by a moderate fall in

eosinophils I don t know anything about mice We have not worked with them but we have given epinephrine to hypophysectomized rats many times and have not seen a significant fall in the eosinophils I think that agrees with Dr Recant s work Speirs and Meyer also reported work on mice (62)

Sayers There is a depletion of adrenal ascorbic acid following hemorrhage exposure to cold administration of histamine or insulin in adrenal demedullated rats Apparently the adrenal medulla is not essential for the accelerated rate of discharge of ACTH which accompanies these stress stimuli that is if one accepts adrenal ascorbic acid as an index of pituitary activity Transplant studies have been conducted in Dr Selye s Dr Long s laboratory as well as in our own Transplanted adenohypophyseal tissue discharges ACTH in response to stress The experiments definitely indicate that direct neural or neurovascular connections between the hypothalamus and the adenohypophysis are not essential for the discharge of ACTH in stress I must point out however that it is possible although not very probable that a neurohumor from the hypothalamus could pass by way of the general circulation to the anterior chamber of the eye the site of the transplant

Thorn An eosinopenic factor from the hypothalamus has been obtained in a relatively crude state

Sayers Was this factor assayed in intact or in hypophysectomized animals?

Thorn Intact animals I am certain inactivating the material and injecting it induces no response

Sayers Such an assay in intact animals means very little The animal with intact adenohypophysis discharges ACTH in response to a great variety of substances I cannot think of a test which would be suitable for the assay of the eosinopenic factor from the hypothalamus

Astwood I think Dr Hume has demonstrated an eosinopenic effect from hypothalamic extract in the animal with the hypothalamic lesion is that right?

Thorn I cannot say

Sayers Recently Dr Long and his group have demonstrated that the direct application of epinephrine to an anterior chamber transplant of the adenohypophysis will bring about the discharge of ACTH This is a valuable contribution to the field It has been interpreted by Dr Long to mean that endogenous epinephrine secreted in response to the great variety of nonspecific stresses has a direct action on the adenohypophysis to induce discharge of ACTH However it is my opinion that other vasoconstrictor agents for example histamine (a vasoconstrictor in the rat) should be applied directly to a pituitary transplant before

generalizations are made as to the physiological role of epinephrine in pituitary regulation

White Are there any suggestions from the group as to how one would assay hypothalamic material

Long I don t know You would require an animal with an intact hypophysis to assay it Otherwise there is always the possibility of a nonspecific response which is not inconsiderable after the injection of any crude tissue extract

Thorn I believe that the fact that extracts made from anatomically related areas produce no eosinopenia does not settle the argument but does make it more likely that a highly active extract comes from a particular location I have only mentioned part of the experiments of Dr Hume in trying to locate the factor The interesting fact is that one does obtain an active material in an extract of one section of the stem and inactive material in an extract from tissue at another point of the stem

Long I think Dr Hume s work has raised some interesting possibilities but we cannot agree with all his findings particularly on the lack of effect of epinephrine after certain hypothalamic lesions This seems to be an unusual finding—one that we have not been able to confirm We have found in rats with lesions produced with the Horsley Clark instrument in approximately the same area as in Hume s experiments that we still get a response to epinephrine Our experience has been that you cannot block the effect of epinephrine in this way This is a difference between the two laboratories that needs to be resolved

Thorn Don t you believe that there is a good chance that the dose level is most important? There appears to be a direct effect of epinephrine on the anterior pituitary but a very much smaller dose of epinephrine than is customarily used in the test may be effective in the intact animal When the hypothalamus is eliminated it may require a much larger dose to obtain a direct effect

Long I would also like to point out something else about these hypothalamic lesions They appear to be located in the area in which the so called epinephrine secretory center has been placed You have to remember that the reflex secretion of epinephrine involves a long pathway the afferent side going up the spinal cord to the medullary centers and then on to the so-called head ganglia in the hypothalamus region while the efferent pathway travels back down the spinal cord to the splanchnic outflow and hence to the adrenal medulla This was pointed out by Elliott in 1906 and repeated by many experimenters (48 63) This is the area in which are located the cells that are the final reflex pathway for the secretion of epinephrine It may well be that the hypothalamic lesions in the experiments of Hume and

Wittenstein (51) have destroyed the cells associated with the reflex secretion of epinephrine and hence have prevented, by interference with reflex epinephrine discharge the usual response of the anterior pituitary adrenal cortical system to cold, trauma, etc In view of our experiments with rats in which the cord was transected at about the level of the third dorsal vertebra it is difficult to believe that any neural or neurohumoral pathway not involved with epinephrine discharge, is concerned with ACTH release You may recall that in these spinal animals a painful stimulus given above the level of the spinal transection did not produce an eosinophilia while a similar stimulus to an animal with an intact spinal cord did so in one hour Obviously, in the spinal animals we have not interrupted any pathways, neural or neurohumoral from the hypothalamus to the anterior pituitary but we have interrupted the reflex pathway for epinephrine secretion It does not matter where one interrupts whether in the hypothalamus the spinal cord or adrenal medulla the pattern of response of ACTH secretion is modified

Dougherty Dr Cartwright (64) has evidence that turpentine administered to adrenalectomized dogs produces a significant fall in eosinophils, but at the same time there is a significant increase in the number of lymphocytes Epinephrine however produced a lymphocytosis but no eosinopenia in adrenalectomized dogs

Thorn I am certain that there is an effect of epinephrine on the eosinophils inducing eosinopenia which is independent of the liberation of adrenal cortical steroids We have observed a fall in certain patients with well demonstrated Addison s disease as well as in patients subjected to bilateral complete adrenalectomy for hypertensive vascular disease

In Dr Long s experiment the effects were not based on a change in eosinophils One s experiments are independent of eosinophil change when the ascorbic acid depletion method is used If one uses the eosinophil fall as an indicator system one still must check with a more direct method I am certain that an eosinopenia following ACTH administration is a highly reliable indicator of adrenal cortical activation of 11 17 oxysteroids I do not feel that the eosinopenia following epinephrine administration has nearly this degree of reliability

Sayers I should now like to discuss the role of the sympathoadrenal system in the regulation of the pituitary adrenal system I would like to warn you that my own ideas regarding regulatory mechanisms have influenced me very definitely in the presentation of this subject It seems to me that epinephrine could act directly on the adenohypophysis It also could act like other nonspecific stresses to increase the rate of utilization of cortical hormones When I use the word

utilization I realize that I am on very shaky ground indeed because we do not know very much about utilization or degradation of cortical hormone Epinephrine could also be the denominator common to all types of stress which promotes utilization of cortical hormone by the peripheral tissues In that case the completely sympathectomized animal would be expected to be less responsive than normal to the metabolic and other actions of epinephrine

Frequent association of epinephrine discharge and ACTH discharge indicates that epinephrine supplements and even makes a major contribution to the excitation of the pituitary adrenal cortical system in a number of stresses For instance in the case of painful stimuli I am sure that a major factor concerned in the excitation of the pituitary adrenocortical system is the discharge of epinephrine from the adrenal medulla Measures which interfere with the discharge of epinephrine lead to various degrees of reduction in the rate of acceleration in the discharge of ACTH in response to certain types of stress However it seems to me that experiments concerned with the blocking of ACTH *discharge do not contribute to our understanding of the mechanism of* the action of epinephrine in inducing discharge of ACTH They are simply concerned with interruption of the sympathoadrenal system itself They do not tell us how epinephrine acts to excite the adenohypophysis

Vogt s claim that epinephrine has a direct action on the adrenal cortex has not been confirmed by Long Furthermore Hechter has shown that ACTH but not epinephrine stimulates the isolated perfused adrenal to produce increased quantities of chemocorticoids The pituitary action of epinephrine or a variety of other stresses is not blocked by Dibenamine ergotamine or TEA These are negative experiments They do not rule out the possibility that epinephrine has a direct action on the adenohypophysis It is of some interest that Sawyer and co workers have demonstrated that Dibenamine will block the copulation induced release of the ovulatory factor from the adenohypophysis If epinephrine stimulates the pituitary directly to release both gonadotropins and ACTH then one is Dibenamine sensitive the other Dibenamine insensitive

The adrenal ascorbic acid index of ACTH activity indicates that adrenal demedullation blocks the pituitary response to sciatic nerve stimulation but not the response to a number of other types of stress such as cold hemorrhage insulin or histamine

An experiment which seems to me very significant is the one in which the action of epinephrine is blocked by pretreatment with cortical hormone It has led me to the conclusion that epinephrine acts indirectly on the adenohypophysis

Loeui As we don t know by which mechanism lack of cortical hormones in the blood stimulates ACTH secretion it would be premature to contend that the hypophysis in a state where it does not react to other stimuli should still react to epinephrine

Sayers It seems to me that if epinephrine had a direct action on the adenohypophysis that action should not be seriously affected by the concentration of cortical hormone in the surrounding medium Yet we know that pretreatment with cortical hormone will block the stimulatory action of epinephrine on the pituitary Let me put it another way You could make out a case for histamine or many other drugs such as acetylcholine as having a direct action on the adenohypophysis These substances stimulate the adenohypophysis yet we do not assign them a special regulatory role!

Long I do not follow the argument that because the adrenal cortical steroids in some way affect the hypophysis conditioning its response to stimuli you can therefore exclude *epinephrine* as a stimulating agent because its effect is also blocked

Sayers How do you visualize epinephrine acting on the adenohypophysis?

Long I cannot make a suggestion in the case of epinephrine and I think very few people can state specifically how any agent brings about a secretion

Loeui Have you any idea as to how lack of cortical hormones stimulates the hypophysis?

Sayers No I am coming to that point in a minute I agree that is a very vague aspect of the subject It seems to me that if pretreatment of an animal with cortical hormone blocks the action of epinephrine and other stresses it has a great deal of significance in regard to the regulatory mechanism under discussion

Long If nothing has an effect after administration of adrenal cortical steroids why are we bothering to look for any stimulating agent to the anterior pituitary other than the blood levels of these substances?

Sayers In the animal not treated with cortical hormone epinephrine does bring about a discharge of ACTH Epinephrine however does not assume a more important role than other stresses which can be blocked such as histamine cold typhoid toxin etc

Long Would you consider that the stress induced by crushing an animal s leg is the same as giving epinephrine? The former is also blocked by cortical extract but one is physiological and the other non physiological

Sayers The important point is that ACTH will stimulate the adrenal cortex in an animal pretreated with a dose of cortical hormone that will block the action of epinephrine

Dougherty It seems to me that the core of your hypothesis concerns the utilization of the hormone If you supply hormone so that you are *equilibrating the amount of epinephrine* with the amount of cortical hormone required by the stress you will have no discharge of secretions of the adrenal cortex To me argument is rather futile until we know whether stress uses up hormone and whether the amount used is directly proportional to the degree of stress

Sayers I am coming to that point in a minute It is an aspect of the problem about which we know very little indeed an aspect which requires a great deal more study

Pincus You previously made the point that severe stress will break through this cortical hormone block? If you were to administer epinephrine in the presumed physiological doses that occur under severe stress would you get this break through?

Sayers The doses of epinephrine we used were greater than the amount expected to be discharged during stress

Thorn A small quantity of adrenal cortical hormone will block the effect of physiological doses of epinephrine in man A point that interests us is that repeated doses of epinephrine have little or no beneficial effect in rheumatoid arthritis I am certain that if epinephrine or some other neurohumoral agent had a direct action on the *adenohypophysis which was independent of the level of circulating* steroids one ought to be able to induce a marked degree of hypercorticism by prolonged administration of epinephrine

Ingle We have some observations which relate to this problem It is true as Dr Long says that epinephrine can cause adrenal cortical hyperplasia but when we have given it over a period of several weeks by continuous subcutaneous injection the cortical enlargement is very slight as compared to the hyperplasia caused by ACTH given continuously Epinephrine loads which cause death may induce considerable hyperplasia just as does any toxic agent but smaller doses which do not kill may still cause marked pharmacologic changes without extensive hyperplasia of the adrenal cortices It is interesting to note that the prolonged administration of epinephrine does not cause regression of the adrenal medulla One of the metabolic changes caused by epinephrine is a decrease in carbohydrate tolerance This is in part at least extrahepatic It might be thought that the decrease in glucose tolerance caused by epinephrine represents hypercorticalism but this response to epinephrine is full blown in the adrenalectomized animal

Conn A clinical problem that falls logically in this discussion is posed by a patient with severe asthma who is epinephrine resistant but responds well to ACTH The possibility is that rapid hypophyseal exhaustion with respect to ACTH has been caused by epinephrine

Long May I point out one thing in regard to the action of epineph rine? Dr Sayers has presented perhaps not conclusive evidence but certainly highly suggestive evidence that there is some relationship between the blood level of cortical steroids and the capacity of the pituitary to discharge ACTH Now if you interpose in the system a substance such as epinephrine it will augment the secretion of ACTH That will result in the release of ACTH raising the blood level of cortical hormone This in turn will at a certain point inhibit ACTH secretion So you can say epinephrine is not a substitute for adrenal cortical steroid and it is not a substitute for ACTH You are working in a mechanism that is inherently a feedback mechanism If you aug ment it temporarily you will raise the level of hormone If Dr Sayers hypothesis is correct you will then dampen down the ACTH secretion thus restoring the original blood level of cortical hormone

Thorn Everything is true except the last part of the statement When one recalls the sustained increase in hormone output one obtains under stress conditions there must be a marked difference between this and the hormonal response following the use of epinephrine The mech anism which you postulate is good but certainly in the case of surgical operation or exposure to other stresses one does not observe exhaustion of the pituitary after four to eight hours

Long So far as I know neither I nor anybody else has said that the only way in which you can get ACTH secretion is by the liberation of epinephrine That would be foolish if for no other reason than that following demedullation of the adrenals of an animal regeneration of the cortical tissue occurs Therefore we must suppose there are other mechanisms associated with the secretion of ACTH I have never suggested that the sole way to augment the secretion of ACTH is by release of epinephrine Perhaps later on I will have an opportunity to enlarge on that particular point I think it should be clear that we do not want to put ourselves on opposite sides of the fence where one says you have to have epinephrine secretion or no ACTH secretion and the other which says you do not need it

White May I ask Dr Long if in his experiments with Dr McDer mott in the direct application of epinephrine the direct application of other substances was tried?

Long We are engaged in that now and we are particularly inter ested in the effects of histamine

Dougherty May I mention in relation to the epinephrine effect in asthma that epinephrine has a therapeutic effect through its capacity to relax bronchiolar smooth muscle whereas ACTH may be acting on the fundamental disease process itself Unless we can evaluate the extent

of these two actions how can one assume that any of the effect of epinephrine in asthma is mediated by adrenal cortical secretions?

Thorn Does not all this discussion bring out the interesting point that epinephrine is a physiological substance which does cause the pituitary adrenal discharge but in contrast to stress conditions it seems to be shut off rather quickly? There are patients with pheochromocytoma who secrete tremendous amounts of epinephrine or nor epinephrine for periods of months or years and one rarely observes changes suggesting adrenal cortical activation

White There may be an organ in the animal which may inactivate epinephrine and which may increase its rate of inactivation when necessary This sounds teleological but there could be a balance between production and destruction The fact that one may have a continued secretion of excessive amounts of epinephrine may not mean that the effects of that epinephrine may continue to be manifested

Long There is no doubt about this There is such a thing as the development of resistance to epinephrine Continued administration of epinephrine raises the tolerance of the animal to doses which in the first administration would kill it in a few minutes

Sayers There is one important experiment which should be mentioned Dr Thorn and his colleagues have shown that the completely sympathectomized dog shows a normal eosinopenic response to formaldehyde It appears that neither epinephrine nor sympathin is essential for discharge of ACTH

I will now attempt to summarize There is a considerable body of evidence to support the concept that the titer of cortical hormone in the body fluids has a regulatory influence on the adrenocorticotropic activity of the adenohypophysis Other factors may modify this regulatory system Dr Long has adequately summarized the experimental evidence in favor of the possibility that epinephrine has a direct action on the adenohypophysis

The thesis that the titer of cortical hormone regulates pituitary ACTH activity has certain deficiencies We have not been able to block completely the discharge of ACTH which follows severe stress The mechanism may apply only to mild and moderate degrees of stress In severe stress anoxia or tissue toxins may have a direct action on the adenohypophysis Likewise very large quantities of epinephrine may have a direct action on the adenohypophysis

The concept that the titer of cortical hormone in body fluids regulates the secretion of ACTH fits rather nicely with certain experimental findings of Dr Ingle When the depancreatized rat is subjected to stress glycosuria is reduced It is difficult for me to see why there should be a reduction in glycosuria if such an animal were subjected to

hypercorticism The result is more compatible with the notion that stress increased the rate of utilization of cortical hormone and resulted in a relative deficiency of the steroid

I am interested to learn why a stress such as epinephrine administration, is not of more therapeutic benefit in the treatment of rheumatoid arthritis than has been reported in the clinical literature If stress induced hypercorticism then it should be an effective substitute for ACTH or cortisone

The concept regarding cortical hormone titer and pituitary ACTH activity does not give us an answer to the problem as to how the cortical hormone acts on the adenohypophysis It could be a direct action of the cortical hormone It could be a metabolic product of the action of cortical hormone at the periphery It could be a metabolic product of a deficiency of cortical hormone It is obvious that the cortical hormone titer thesis is built on very indirect evidence In addition it leaves many questions to be answered

Astwood Dr Thorn s suggestion that cortisone may inhibit the adrenal cortex directly is interesting There are many points that are attractive about it to explain the extraordinary refractoriness of the cortisone treated patient to ACTH It occurs to me though that if the adrenal cortical secretion did inhibit the adrenal cortex directly then ACTH too should become ineffectual with continued administration, yet it does not seem to

Loeb Might not that depend upon the rate of utilization of cortical steroids at the periphery somewhere?

Astwood If cortisone is given in a dose just sufficient to produce features of Cushing s syndrome and if ACTH is given after that it would not be effective Now if the same degree of hypercorticism were induced with ACTH the excessive cortical secretions would not in turn make the ACTH ineffectual This suggests that cortisone does not act to depress the adrenal directly Is that right?

Sayers I have no evidence which has a direct bearing on the question A single dose of ACTH rapidly disappears from the blood stream Yet as far as chemical indices are concerned the action of the hormone persists for three or even six hours Despite the fact that ACTH can no longer be detected in the blood metabolic processes in the adrenal cortex and in the organism indicate that the gland continues to be active

Thorn I believe you can go one step further You do not have to postulate that ACTH has anything more than an instantaneous effect on the adrenal If you give a dose of cortisone intravenously and follow the rate at which the eosinophils disappear you observe the same rate of fall as following an injection of ACTH I would say in answer to

Dr Astwood that we often see loss of effect on continued use of ACTH but there are so many intermediary factors that it is impossible from our viewpoint to say which of them is the primary factor a) resistance to antibody formation b) increased local destruction at the site of injection and c) depletion of adrenal reserves

Conn Dr Wolfson (65) has just reported that patients receiving a constant dose of ACTH over a long period of time frequently show a diminishing response to it both from the clinical and metabolic points of view Wolfson found under those circumstances that uptake of radioactive iodine was diminished and that protein bound iodine of serum was diminished He concluded that thyrotropin was inhibited by the adrenal steroids a conclusion which Dr Thorn and others had arrived at earlier When Wolfson added desiccated thyroid to the regime using the same amount of ACTH the metabolic responses rose promptly This suggests that the adrenal cortical cells were suffering from myxedema so to speak and were unable to respond as well to a given dose of ACTH as they were when they were made more receptive or responsive by the addition of desiccated thyroid

Astwood We have not seen a clear case of unresponsiveness developing to the ACTH preparations which we have used I wonder whether apparent refractoriness may not be due to differences in the potency of different batches The preparations may be different

Thorn It might take more than one hundred to two hundred cases before one would run into this complication We did not observe unresponsiveness until the second year of our studies on ACTH We have definite indications that three changes may occur which will reduce the activity of ACTH a) thyroid inhibition with long continued ACTH and adrenal steroid stimulation making doses of ACTH less effective because of the concurrent hypothyroidism—such an alteration can be modified by the administration of cortisone b) increased destruction of ACTH at the site of intramuscular injection under which circumstances intravenous infusion of ACTH is still highly effective c) true antibody formation to specific protein

Astwood Has anybody ever seen the clinical picture of myxedema in Cushing s syndrome?

Thorn These patients have a very low uptake of I 131 If one is looking for hypothyroidism it is not as easily detected by a basal metabolic rate as by a depression in serum protein bound iodine a reduced rate of radioactive iodine uptake by the thyroid and by the clinical picture

Conn The protein bound iodine is down?

Thorn Yes it is low The patient appears as a very flushed individual in whom one might not expect to find reduced thyroid activity

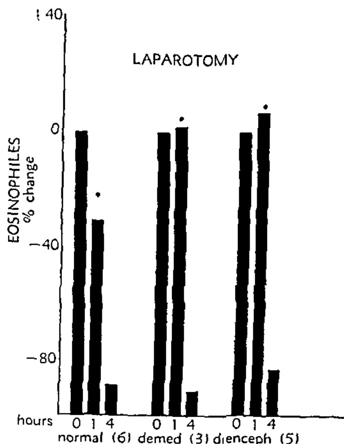


FIGURE 8*

Long I would like to present three figures which may help in this discussion It seems to me one factor that has not been brought out so far is the time relationship of the response I think that this is important and Dr Sayers touched upon it

In Figure 8 you will see the effects of laparotomy with manipulation of the intestines in three types of animals In the normal animal this procedure is followed by a well marked fall in eosinophils in one hour and an extreme fall in four hours On the other hand a similar procedure carried out on the rat with demedullated adrenals shows that in one hour there has been no change in the eosinophil count, although

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* Reprinted from *Yale J Biol & Med* 23 5[illegible] (1950)

there is again an extreme fall by the fourth hour In animals with suitably placed lesions in the diencephelon a similar pattern of response to laparotomy is seen We interpret this to mean that the reflex pathway for the secretion of epinephrine has been interrupted either by demedullation of the adrenals or by destruction of the ganglia in the brain stem that are concerned with this reflex Similar results are observed in animals in which the spinal cord has been transected some weeks before at the level of the third dorsal vertebrae

I would like to emphasize that although interference with the reflex secretion of epinephrine abolishes the immediate response of the eosin

PROPOSED MECHANISM of CONTROL OF THE SECRETION of ADRENAL CORTICAL STEROIDS

FIGURE 9*

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*Reprinted from Yale J Biol & Med 23 52 (1950)

ophil level to trauma it does not prevent a much more slowly acting mechanism from operating to lower this count

I may add that a similar alteration in the period of response to insulin hypoglycemia or to histamine injection is brought about by demedullation of the adrenals We may therefore assume that there exists another mechanism over and above that initiated by epinephrine release whose characteristics are first the slowness of its response and, second its persistence once a response has been initiated

For my part I doubt if one hypothesis is sufficient to explain the mechanism of ACTH release under the diverse and different circum stances that it occurs particularly when one considers the rapidity of its release Rather it would seem that we are observing the interplay of two connected mechanisms one a self regulating humoral system involving merely the relative blood concentrations of ACTH and adre nal cortical hormone the other representing a part of the response of the autonomic nervous system The former is probably a much more primitive system than the latter The activation of the autonomic nervous system with the release of its reinforcing hormone, epineph rine is able to bring about with great rapidity the release of ACTH and hence of cortical hormone to meet the immediate requirements of the organism while the purely humoral system comes more slowly into operation and may not even be operative at all if the pressure on the organism is of short duration

Figure 9 illustrates our present concept of this dual regulation of the pituitary adrenal cortical system For want of more appropriate terms we have called the mechanism proposed by Sayers the metab olic phase and that involving stimulating of the autonomic nervous system with release of epinephrine the autonomic phase

As we have remarked in a recent publication these phases may be independent or sequential depending on the conditions of stress en countered by the organism

In conclusion I would like to point out that the mechanism proposed above is in many respects an extension of the emergency hypothesis formulated by the late W B Cannon (66) to explain the physiological events that follow the release of extra epinephrine when the organism is called upon for unusual efforts to resist threatened and real dangers to its existence If the above proposal has any merit we must now add the activation of the anterior pituitary adrenal cortical system to the responses that have previously been shown to be associated with this hormone This would seem to be a fitting extension of Cannon s views since increased quantities of cortical hormone are essential to meet many circumstances and indeed may be the most crucial factor in the success or failure of the adaptive processes

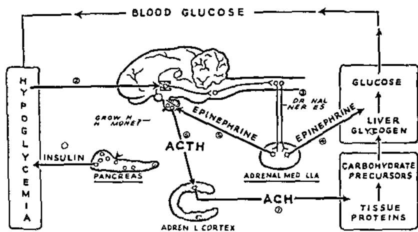


FIGURE 10

Consider for example the well known effect of insulin hypoglycemia in increasing epinephrine secretion—an effect first directly demonstrated by Cannon and his colleagues (See Figure 10) They believed that such a release of epinephrine counteracted the hypoglycemia by accelerating the conversion of liver glycogen to glucose In light of what has been said today we must also realize that such a release of epinephrine also activates the pituitary adrenal cortical system leading to the release into the bloodstream of increased amounts of the adrenal steroids which have as one of their effects the accelerated conversion of tissue proteins to liver glycogen and hence continually adds to the amounts of glucose available for counteracting the hypoglycemia

Recognition of the involvement of the adrenal cortical hormones as well as epinephrine in the response of the organism to hypoglycemia gives us a clearer understanding of the well known fact that even fasted animals with minimal quantities of liver glycogen can recover spontaneously their blood glucose level after moderate doses of insulin and accounts at least in my mind for the failure of hypophysectomized animals to accomplish this

Thorn I was impressed this past summer in talking to Professor Hess in Zurich regarding the nature of the cells in the hypothalamus They are actually quite primitive The fact that they are not typical highly developed nerve cells appears to give them potentialities for secretory activity This is very interesting in connection with our present concept of a hypophyseal portal system of veins and the fact that

polyethylene film placed between the hypothalamus and pituitary does not block stimulation of the pituitary This can be interpreted as indicating that there is a hormonal transmission of substances from the hypothalamus which may be an important factor in the release of ACTH from the pituitary

Long You are still thinking of something coming from the hypothalamus?

Thorn Yes, to the pituitary

Long Why do you get a difference of response when you interrupt epinephrine secretion?

Thorn I think that is explained as you have shown in Figure 9, but I wonder whether it is the whole story

Ralli We have found that the effect of ACTH as measured by the response of the white blood cells and eosinophils could be conditioned by certain nutritional substances This has been true both in the intact and the adrenalectomized animal The nutritional factors that we have studied are pantothenic acid and B 12 Intact rats fed a diet deficient in pantothenate for thirty days show only a slight eosinopenia at two four and six hours When the diet is supplemented with large doses of pantothenate for seven days following the period of deficiency the eosinophiles decrease significantly at two and four hours after the ACTH The response as a matter of fact is almost normal When ACTH is injected into adrenalectomized rats and the diet has been deficient in pantothenic acid there is a very marked increase in the eosinophils at the fourth hour and the count is still significantly high at six hours If these adrenalectomized rats are given pantothenate in large doses for seven days following the adrenalectomy the increase in eosinophils is sharply curtailed and there is practically no change in the eosinophil count following the injection of ACTH When adrenalectomized rats receive large doses of B 12 an eosinopenia occurs following the injection of ACTH These findings suggest to us that the action of the hormone depends to a certain extent on the situation within the cells and this apparently can be influenced by excessive amounts of certain nutritional factors

Thorn If you remove the adrenals in mice you continue to obtain an eosinopenic response to ACTH In addition to adrenalectomy it is necessary to castrate the animals since there appears to be tissue in the gonads which responds in a fashion similar to the adrenal cortex

Ralli In the experiments that we are reporting the sex of the animals did not seem to affect the response

Long As far as the eosinophil change is concerned if you have the gonads present there is still a slight response to epinephrine in adrenalectomized rats It is not until you carry out castration as well as

adrenalectomy that you completely obliterate the response to epinephrine It may imply that there is an overlapping in steroid action here In the normal hypophysectomized rat you obtain an increase in eosinophils In the adrenalectomized rat there is a slight fall in the first hour

I would like to emphasize one other thing when talking about epinephrine, that is the quantities involved I have no doubt if large quantities of epinephrine are given to an adrenalectomized or hypophysectomized animal you may have toxic effects It is well to remember that the effect of epinephrine on the adrenal ascorbic acid can be brought about by amounts that barely affect the blood glucose level and certainly are below those that will cause any significant pressor responses Apparently you do not need large quantities of epinephrine to stimulate the pituitary mechanism The amounts required are within the physiologic capacity of the adrenal medulla to secrete under any circumstances

Thorn We have studied the synergism between epinephrine and adrenal steroids using the eosinophil response as an index Simultaneous injections of doses of epinephrine and doses of ACE or cortisone which separately have no effect on the eosinophils will cause a marked eosinopenia

Pincus In the Addisonian?

Thorn In the Addisonian patient in whom there is no endogenous medullary or cortical hormone There are other evidences of potentiation of these two hormones

Loewi In connection with the question regarding the release of a hypothalamic substance it might be of interest to you that Harris quite recently mentioned the possibility that this substance might be epinephrine like Accordingly epinephrine would originate from two sources

Astwood In connection with the hypothesis that the pituitary receives blood which drains down from the hypothalamus I think Dr Wislocki pointed out in the original description that this venous system arose in the meninges not in the hypothalamus Is that correct?

Pincus Dr Sayers I am interested in the question of utilization particularly as it relates to specific steroids You have studied adrenal extract and cortisone and desoxycorticosterone acetate? Have you done work with any other types of corticosteroids?

Sayers Yes we have studied cholesterol estrogen progesterone desoxycorticosterone corticosterone 17 hydroxycorticosterone cortisone aqueous adrenocortical extract and lipoadrenocortical extract

Pincus All blocking agents?

Sayers Cortisone and 17 hydroxycorticosterone were the most potent blocking agents Corticosterone and desoxycorticosterone were less

potent Progesterone had a slight inhibitory effect at large dose levels Cholesterol and estrogen were inactive

Pincus How did the adrenal cortical extract compare to cortisone in terms of biological activity? In other words was it equal milligram per milligram in terms of biological activity?

Sayers I am sorry I cannot give you an answer because crystalline compounds and adrenocortical extracts were not employed in the same series

Pincus Did you have any idea?

Sayers I can give you a rough estimate Exposure to cold (4° C) for one hour required about 0 2 ml of adrenocortical extract whereas in the case of the 17 hydroxycortisone it required about 50 mcgm

Pincus What extract?

Sayers Upjohn s aqueous adrenal cortical extract

Pincus Do you know the cortisone equivalent of 2 ml of aqueous extract?

Ingle The expression cortisone equivalent does not mean the actual cortisone content per 1 ml of adrenal cortex extract but refers to the amount of cortisone required to cause the deposition of as much liver glycogen under standard conditions as 1 ml of extract One milliter of Upjohn s beef adrenal extract has the activity equivalent of approximately 0 2 mg of cortisone

Pincus So it is very close

Thorn We thought that by using adrenal extract and studying the fall in eosinophils in Addisonians that we had devised a quick method for assaying the content of 11 17 oxysteroids in extract until we dis covered that one extract always gave a definite though small fall in eosinophils Other adrenal extracts which appeared to be equally effective clinically did not produce an eosinopenia It is of interest that the extract which caused the eosinopenia was found to have an appreciable quantity of epinephrine in it Thus one cannot use the eosinophil fall in patients with Addison s disease as a measure of 11 17 oxysteroids unless one is certain that there is no epinephrine present in the extract On the other hand as far as crystalline prepa rations are concerned the method of assay is an excellent one

Long Is it not true to say that the Cori Cycle may need cortical hormone to function properly? I have in mind Secker s experiment (67) with the nictitating membrane in which the response to epineph rine was greatly diminished in the adrenalectomized animal On injecting cortical hormone a normal response was obtained It may well be that in adrenal insufficiency you do not get a normal response to epinephrine as you do not get normal response to a number of other things

Astwood I am sure Dr Sayers is always asked this question in regard to his theory that a drop in circulating cortical hormone consequent to stress stimulates the secretion of ACTH How does he explain the fact that stress leads to an excess quantity of cortical hormone in the urine?

Sayers That is difficult to explain Presumably the excertion in the urine reflects the levels in arterial blood

Astwood If utilization is the main stimulus then you would expect the level to be low in stress

Long I might point out that the adrenal cortical hormones are secreted into the venous blood and mixed with the heart blood If the pituitary received only arterial blood you would think you would very soon balance the increased utilization of cortical hormones by the increased output

Sayers Adrenal vein blood enters the *vena cava* and is carried to the right heart From there it goes to the lungs and then into the left heart and out into the aorta

Li I should like to ask Dr Sayers about the rate of disappearance of cortical hormone in the body What is the rate of disappearance in *normal stressed animals?*

Sayers The relative rate of disappearance of cortisone under these two conditions would be of some interest but we have no data at present

Ingle According to Dr C C Porter and R H Silber of the Merck Institute * cortisone given intravenously in the mouse disappears very rapidly under resting conditions Within a few minutes most of it disappeared and was not to be found anywhere

Thorn I would be very much surprised if the stress increased the rate at which cortisone disappeared since its disappearance from the blood stream is so rapid under normal circumstances

Sayers Let me ask you this question Dr Thorn Adrenalectomized animals under optimal conditions of environment require very small amounts of cortical hormone to maintain a state of well being However application of stress increases the requirement markedly?

Thorn That is right

Sayers How do you look upon this difference in requirement? What is happening under these circumstances?

Thorn I would look on it now as being necessary for intensive metabolic activity although that is quite undesirable In the nonstress condition one can cure or relieve patients with rheumatoid arthritis with a given level of hormone I do not believe that Dr Bauer would

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P l comm ion

subscribe to the idea that the rheumatoid arthritic would utilize cortisone more rapidly than the normal Would you Dr Bauer?

Bauer No

Thorn The only evidence we have is that we do produce a change in inflammatory reaction If one wishes to block an inflammatory reaction, it often requires a level of hormone great enough to produce Cushing s syndrome If the increased utilization were a factor, you would never produce Cushing s syndrome In anoxia or stress it has been shown that the hormone requirement is greatly increased by raising the altitude to which an animal is exposed but that if the new altitude is sustained the quantity of hormone required by an adrenalectomized animal in the new environment may not be appreciably increased over that of lower levels

Sayers There is another interpretation of that experiment You can interpret it to mean that the reduction in requirement for cortical hormone with continued exposure is associated with adaptation of the animal When the homeostatic mechanisms concerned with the resistance of the animal to low tension of oxygen come fully into play then the high altitude is less of a stress and less cortical hormone is required

Pincus You should have one of the answers because surely you have studied 17 ketosteroid excretion following cortisone administration to Addisonians as compared to arthritics or other patients Is there any difference?

Thorn Not that I can tell within the limits of validity of interpretation because one always has the suppression of the adrenal in a patient with intact adrenals and the over all effect on 17 ketosteroid excretion must include this factor whereas in the female patient with Addison s disease all changes in 17 ketosteroids can be interpreted in relation directly to the material given In other words if one observes no change in 17 ketosteroid excretion when one administers 75 mg of cortisone to a patient with intact adrenals that may indicate that the hormone is reducing the endogenous adrenal activity

Pincus In the acute experiment or is this a practically immediate effect on endogenous ACTH?

Thorn We have always used the intramuscular material I believe that with further studies on orally administered hormone you might be able to answer that question

Astwood In connection with what Dr Thorn said concerning the matter of increased utilization of cortical hormone under stress I think it is true that a person who is severely ill will develop the features of Cushing s syndrome with a dose of cortisone or ACTH which will also produce it in the normal person Indeed it has seemed to us that people who are seriously ill tend to develop Cushing s features sooner

than relatively healthy people If the stress were using up the hormone they should not show features of Cushing s syndrome so readily

Ralli Do you interpret that Dr Astwood as meaning that perhaps the physical state within the cell or the intracellular substances was having something to do with the reaction?

Astwood I was only interpreting it to mean that seriously ill patients do not seem to use up the hormone any more quickly than the normal person I do not know what it means beyond that

Thorn It would appear feasible to answer this by giving increasing doses of cortisone at the same time that one applies stress to find out how much cortisone is required quantitatively to block the stimulation from stress Has this been tried? If so what were the results?

Sayers We found that when doses of histamine of 0 25 0 5 and 1 mg per 100 gm of body weight were injected into rats increasing the dose induced corresponding increases in the degree of depletion of adrenal ascorbic acid The more severe the stress the greater the degree of depletion In the case of 0 25 mg of histamine 20 mcgm of cortisone per 100 gm of body weight of rat completely blocked pituitary discharge of ACTH However 20 mcgm is entirely inadequate for 0 5 mg of histamine per 100 gm body weight This dose of histamine required 100 mcgm of cortisone to block the pituitary Five hundred to one thousand micrograms gave only a partial block when 1 mg of histamine was administered

Two conclusions may be drawn from this experiment a) the degree of inhibition of pituitary ACTH activity is proportional to the amount of cortical hormone administered b) the greater the degree of stress to which the animal is subjected the greater is the amount of cortical hormone required to inhibit pituitary adrenocorticotropic activity

Loewi There is no doubt that just as the release of gonadotropic and thyrotropic hormones is regulated by the blood level of their respective target hormones also the release of ACTH is regulated by its target hormones This holds true for normal conditions What however is the situation in stress? It has been definitely shown that epinephrine is released in stress and that it directly stimulates the anterior lobe This effect is produced immediately by physiological doses even if smaller than those needed for increasing glycogenolysis in the liver Accordingly I do not see any objection to the concept that epinephrine is the trigger responsible for the release of ACTH in stress

Long Dr Loewi stated the case for epinephrine admirably in my opinion

Sayers I take the position at the present time that in mild and moderate degrees of stress the titer of cortical hormone has a major

role to play in the regulation of pituitary adrenocorticotropic activity Other factors such as epinephrine undoubtedly have modifying influence

Loewi Acting as a trigger?

Sayers Epinephrine would presumably be involved in rapid discharge of ACTH I feel that further experimentation, however is necessary before we can come to the conclusion that epinephrine has a direct action on the adenohypophysis

Loewi What is your opinion of Long s experiments conducted with implants of hypophysis in the anterior chamber of the eye? I feel we have to accept Long s concept as long as it is not disproved

Sayers These experiments should be properly controlled Other agents in addition to epinephrine should be introduced into the site of the adenohypophyseal transplant We have considered the possibility that anoxia and toxins may have a direct action on the adenohypophysis itself

Loewi The main point is that epinephrine acts directly on the hypophysis even if it acts by producing asphyxia

Sayers That is correct

Long I might add that the anterior pituitary transplanted into the eye lends itself quite admirably to studies of Dr Sayers idea because you can put minute crystals of cortisone in with your transplant

Pincus I was going to ask if Dr Sayers ever placed rat pituitaries into a solution of corticosteroid and observed what happened

Sayers We have some experiments in progress I do not like to say too much about the experimental data in this connection because it is an *in vitro* system involving hormones—a most difficult problem indeed Dr Dickman Dr Cheng Mr Merkin and I find that pituitaries obtained from adrenalectomized rats (operated twenty four hours previous to sacrifice) produce ACTH activity during incubation in the Warburg Pituitaries from intact rats actually lose activity during the same period We have conducted eight experiments in all In six of them we have obtained a definite increase in ACTH activity and in two we obtained no increase We are concerned about the reproducibility of the phenomenon and hope to settle this phase of the problem before proceeding to the addition of corticosteroids to the medium

Ingle I have two comments First we have some indirect evidence relating to the amounts of hormones which the adrenal cortices of the rat must secrete under different conditions Under resting conditions we would judge that the adrenal cortices of the sexually mature male rat must be secreting the activity equivalent of 3 or 4 ml of commercial beef adrenal extract per day Under the severe stress of muscle work we have to give not less than 20 ml per rat per day of the same

cortical extract by continuous intravenous injection to obtain optimal work performance If we use cortisone acetate under the same conditions 4 or 5 mg per twenty four hours per rat are required If we give ACTH by continuous injection to normal rats we produce signs of hypercorticalism which require about 10 mg of cortisone acetate per rat per day to duplicate If we give 5 mg of cortisone per rat per day to normal rats under resting conditions we produce some signs of *hypercorticalism and tissue damage We conclude that the adrenal* cortices may undergo very wide naturally occurring excursions in secretory activity During severe stress the adrenal cortices may secrete hormone in amounts which would cause damage if given in the absence of a need

My second comment has to do with the unpublished observations of my co workers Dr B L Baker and Mr William Castor Department of Anatomy University of Michigan Medical School They find that the brains of rats which have received continuous injections of ACTH in our laboratories have cellular atrophy in the periventricular nucleus of the hypothalamus Whether these changes represent specific effects of the hormone and may be related to the mechanism controlling the secretion of ACTH by the anterior pituitary we do not know Similar changes have been described in diabetic animals Our rats given ACTH had hyperglycemia and glycosuria

Ralli What was the work to which you subjected these adrenalectomized rats in order to evaluate the hormone requirement?

Ingle They were anesthetized with sodium phenobarbital and the gastrocnemius muscle was stimulated to lift 100 gm five times per second

Long These changes in the periventricular nucleus I think have been described for several conditions You are probably referring to Von derahe s work (68) on the relation of diabetes mellitus to these changes As I recall there are a great many pathological states associated with pyknotic changes in these nuclei I wonder how specific it is

Ingle It is quite possible that the changes are nonspecific

Long Dr Dougherty will discuss The Relation of the Adrenal Cortex to Hypersensitive States

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THE RELATION OF ADRENAL CORTICAL HORMONES TO THE HYPERSENSITIVE STATE*

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THE STUDIES I am reporting were done in order to evaluate, by quantitative methods the importance of adrenal cortical hormones and hypersensitivity in the various manifestations of allergic phenomena Three experimental approaches were used first the capacity of various adrenal cortical hormones to provide resistance to fatal anaphylactic shock was quantitatively analyzed, second the tissues and organs of hypersensitive animals in anaphylaxis were examined microscopically finally the effects of adrenal cortical hormones on localized inflammations produced by allergic traumatic and chemical phlogogenic stimuli were investigated

It has long been known that adrenalectomized animals are more sensitive to anaphylactic shock than intact animals (1) Although no strong evidence has been submitted indicating that administration of ACTH or adrenal cortical extracts increases resistance to the challenging allergen dose when given to sensitized guinea pigs (2) we have shown that secretions of the adrenal cortex play a major role in the natural resistance of mice to fatal anaphylactic shock (3 4) In spite of the fact that the intact sensitized mouse is the most resistant of animals to anaphylactic shock (5) adrenalectomized sensitized mice were found to be as highly reactive to the challenging dose as sensitized guinea pigs (4) The antianaphylactic potency of adrenal cortical hormones was assayed in terms of their ability to reelevate the resistance of adrenalectomized sensitized mice to the high level of resistance found in intact sensitized animals By means of this method the comparative antiallergic activity of various adrenal cortical hormones can be determined All of the mice used in the experiments on anaphylactic shock whether intact or adrenalectomized were sensitized by subcutaneous injection of 1 2 ml of horse serum in two divided doses twenty one and nineteen days before intravenous administration

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of the challenging dose of the homologous antigen The challenging amount of horse serum given to sensitized intact mice was varied from 0 01 to 1 ml per 20 gm of mouse It was found that normal sensitized mice (CBA) employed in this study were completely unresponsive to any dose from this wide dosage range The LD_{50} of horse serum in sensitized normal mice of strain CBA was thus found to be greater than 1 ml (Table X)

Acute anaphylactic death of sensitized adrenalectomized animals occurred over a wide dosage range when the challenging allergen dose was given two hours after operation The LD_{50} of horse serum in these mice was 0 00052 ml per 20 gm mouse (Table X) Absence of the adrenals was thus found to increase the anaphylactic sensitivity of mice by far more than two thousand times The protective role of adrenal cortical hormones in anaphylactic shock was acertained by giving varying amounts of cortisone at different time intervals prior to administration of the antigen to sensitized adrenalectomized animals The index of protection was 162 when 1 mg cortisone was given two hours before shock In order to restore the resistance of the sensitized adrenalectomized animals to that of the sensitized intact group a total of 2 mg of cortisone was necessary To display this effectiveness this amount must be given in divided doses the first dose two hours and the second dose not more than ten minutes before horse serum administration It is suggested that the first large dose of hormone is essential to saturate the tissues and the second dose given just before antigen administration is necessary to provide a continuous supply of hormone during the period when anaphylactogenic substances are elaborated Thus the second dose may in a sense replace the increased adrenal cortical secretion produced by stress in intact animals

Desoxycorticosterone acetate when given in amounts equivalent to the optimum dose of cortisone acetate provided no protection to anaphylactic shock In fact when equal doses of DCA and cortisone were given simultaneously the degree of resistance obtained was markedly less than that provided by the same cortisone dose given alone This finding is in agreement with the suggestion that DCA antagonizes the action of cortisone directly in tissue rather than via the pituitary (6)

Histamine is considered to be one of the major chemical mediators of anaphylactic shock in several species However the mouse is highly refractory to this substance (7) In order to determine whether histamine might be a mediator of anaphylactic shock in this species sensitized adrenalectomized mice were given the antihistamine pyribenzamine (2 5 mg per kg) fifteen minutes before administration of horse serum This amount of pyribenzamine provided a degree of protection (196 fold) comparable to that obtained in intact guinea pigs for the

TABLE X

Determination of Incidence of Anaphylactic Death in Sensitized Mice

	Groups and Treatments	Dosage of Various Substances	Number of Animals	LD_{50} ml Horse Serum per 20 gm Mouse	96% Fiducial Limits ml Horse Serum per 20 Gm: Upper	96% Fiducial Limits ml Horse Serum per 20 Gm: Lower	Index of Protection*	
I	Adrenex—Untreated	—	323	0 00052	0 0006	0 00044	—	
II	Adrenex + DCA	1 mg /20 gm	69	0 00049	0 0007	0 00036	0 99	
III	Adrenex + Cortisone Acetate (10 min before shock)	1 mg /20 gm	40	0 0065	0 033	0 0014	12 5	12 9 (III and IV)
IV	Adrenex + Cortisone Acetate (2 hrs before shock)	1 mg /20 gm	26	0 084	0 139	0 051	162	
V	Adrenex + Cortisone Acetate (2 doses 2 hrs apart)	2 mg /20 gm	45	>>1 0	?	?	>>2000	
VI	Intact Controls (Sham operated)	—	82	>>>1 0	?	?	>>>2000	
VII	Adrenex + Pyribenzamine (Deaths within 3 hrs)	2 5 mg /kg	217	0 102	0 165	0 0628	196	3 6 (VII and VIII)
VIII	Adrenex + Pyribenzamine (Total deaths within 18 hrs)	2 5 mg /kg	217	0 0285	0 0483	0 0168	55	
IX	Adrenex + Cortisone Acetate (2 doses 2 hrs apart) + DCA (2 doses 14 hrs apart)	2 mg /20 gm Cortisone + 2 mg /20 gm DCA	49	0 225	0 361	0 141	450	
X	Adrenex + Cortisone Acetate (2 doses 2 hrs apart) + DCA (1 dose 2 hrs before shock)	2 mg /20 gm Cortisone + 1 mg /20 gm DCA	35	0 269	0 406	0 178	541	

* Treated dren x ss comp ed to nont e t d adr nex

first three hour period after the challenging dose Subsequently how ever the animals went into a delayed anaphylactic shock so that within eighteen hours after administration of the challenging dose the index of protection had fallen to 55 Nevertheless it is apparent that as in the guinea pig histamine is at least one chemical mediator of anaphylactic shock in the adrenalectomized mouse The occurrence of delayed anaphylaxis suggests that the histamine elaborated at the time of antigen antibody union is prevented from acting by the antihistaminic agent but is still effective when the action of the antihistaminic has ceased This evidence corroborates the previous observations that either destruction or elimination of histamine is decreased in the absence of the adrenal cortex (8 9)

Tissue alterations of mice subjected to anaphylactic shock were confined primarily to the cardiovascular system the loose connective tissue and the bronchiolar epithelium Within fifteen to forty five minutes after horse serum had been given to sensitized animals changes occurred which were confined to the endothelial cells of smaller blood vessels of heart lungs and kidney The changes were degenerative and proliferative and consisted of swelling of endothelial cytoplasm of formation of vacuoles in the nuclei and in some instances of actual proliferation evidenced by the appearance of mitotic figures in the endothelium (3) (See Figures 11 and 12) These early changes are similar to those produced by administration of histamine (10) In large blood vessels splitting of elastic membrane and edema of the media was evident (Figure 13) In addition to the changes in the connective tissue components of the blood vascular system smooth muscle degeneration was found in many of the nonsurviving adrenalectomized animals (Figure 14) Finally edema of the adventitia of the large arteries and of the connective tissue around the bronchioles was observed in the shocked adrenalectomized mice this latter finding is in agreement with that reported by Warren and Dixon (11) The alterations described above were found in varying degrees in all groups of experimental animals Of the alterations in the vascular system only those occurring in the endothelium *were observed consistently in intact shocked* animals

Administration of pyribenzamine prevented alterations in the blood vascular system in animals which survived the shock dose In those animals dying a delayed anaphylactic death however histological alterations of the same extent were observed as in nonprotected adrenalectomized animals The most extreme changes occurred in the shocked adrenalectomized animals given desoxycorticosterone

Bronchiolar constriction edema and desquamation of the epithelial lining of the bronchioles emphysema and reticuloendothelial cell swell











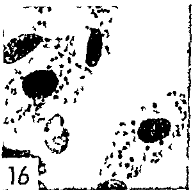


FIGURE 11 Lung Vacuolizat on and swell ng of endoth l al l ng of mall arte y Alt rati s in smooth muscle of the same ve sels F fteen m nutes aft r an phyl ct c hock H E x300

FIGURE 12 Lung Mitot c figure n nd chel m of arte ol F fteen m nut s after anaphyla t c shock H E x450

FIGURE 13 Stomach Alter tion of nt rn l el t c meml ane f ga t c a t ry Twel e n nut after anaphylact c hock Fla t ca ta x300

FIGURE 14 Heart Alte t ns n m tl n set f ll or y y lo ato f th ombus Twel e m ute afte a phyl ct c fock H E x300

FIGURE 15 Loo e connect e t s e Acc mulat o of fib obla t l t lo r) ort sone (1 mg i p) inje tion May Grunw ld Giem 760

FIGURE 16 Loose connective t ssue F brobl t c clu on fo r d) lt o t son (1 g i p) adm n trat on M y Gru wald-G em a x760

ing were all observed in insufficiently protected adrenalectomized animals subjected to shock doses of horse serum The endothelium of the capillary loops of the renal glomeruli showed similar changes to those described previously for other capillaries As might be expected lymphocytolysis was present in lymph nodes spleens and thymi of intact challenged mice but was completely absent in the organs of challenged adrenalectomized animals

The total leucocyte and the differential blood cell counts of the intact adrenalectomized and hormone treated sensitized adrenalectomized mice subjected to anaphylactic shock were also determined at various time intervals after the shock dose Administration of horse serum to intact sensitized animals resulted in a leucopenia lymphopenia, and eosinopenia which occurred within a ten to twenty minute

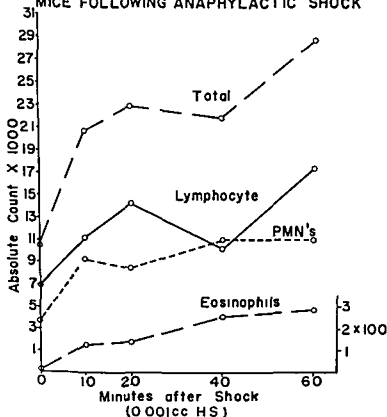


FIGURE 17

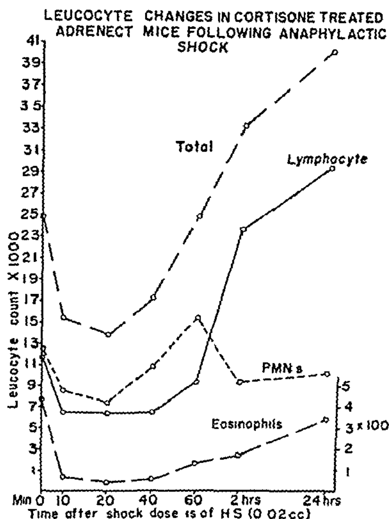


FIGURE 18

interval after stress The presence of a leucopenia following anaphylaxis has been taken as evidence that the histamine appearing in the blood *during shock is released from destroyed leucocytes* (12) *This* rapidly developing leucopenia was not seen in nontreated adrenalectomized challenged animals (Figure 17) but did take place in the *cortisone treated* adrenalectomized mice (Figure 18) These observations indicate that leucopenia does not necessarily accompany anaphylaxis and that occurrence in cortisone and in ACTH treated animals points to a major role of adrenal cortical secretions in the mediation of the leucopenia of anaphylaxis

In summary then it is suggested that the mouse a species virtually

incapable of responding with anaphylactic symptoms at any stage of sensitization to a challenging dose of antigen is as sensitive to anaphylaxis as any other species if it has been deprived of its adrenals This work demonstrates that the adrenal cortex plays a dominant role in ameliorating the clinical phenomena of anaphylaxis The contrast between the highly refractory intact and the highly sensitive adrenalectomized mouse paved the way for a quantitative study The LD$_{50}$ of challenging antigen can be determined within a small range of variation of standard error Thus it was possible to demonstrate that anaphylaxis is not an all or none phenomenon but is a graded response which under uniform conditions of sensitization depends upon the size of the challenging antigen dose and upon the quantitative relation between the doses of antigen and of the protective agent namely the adrenal cortical hormones

It is evident that among the steroid hormones tested so far the C 11 oxysteroids exert such antiallergic action whereas DCA not only does not inhibit the anaphylactic process but actually synergizes it in the sense that it apparently suppresses the protective action of cortisone

The mechanism of action of C 11 oxysteroids in hypersensitive reactions has not as yet been ascertained Adrenal cortical hormones could modify the anaphylactic response by acting in one of several ways a) by inhibiting the union of antigen and antibody b) by diminishing the extent of this union or c) by interfering with the action of the anaphylactogenic substances released by the antigen antibody union

It is apparent that the antigen antibody union is not inhibited and that the production of anaphylactogenic substances is not diminished by adrenocortical hormones because anaphylactic tissue lesions do occur in intact shocked animals and cortisone does not inhibit the Schultz Dale reaction *in vitro* according to Loewe * The fact that pyribenzamine provided a considerable degree of protection against anaphylactic death indicates that histamine is formed during the antigen antibody union in mice The delayed death of pyribenzamine protected adrenalectomized mice indicates that the histamine formed following administration of the challenging dose persists or continues to be released in the tissues of these adrenalectomized shocked animals Apparently then the elimination not only of exogenously administered histamine but also of histamine produced endogenously in allergy is dependent upon adrenal cortical hormones

At present we have no evidence as to whether adrenal cortical hormones suppress formation of or neutralize the action of anaphy

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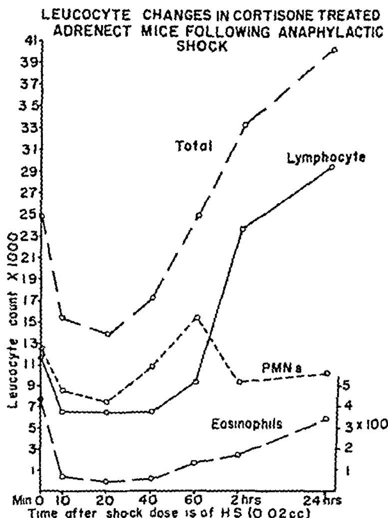


FIGURE 18

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In summary then it is suggested that the mouse a species virtually

ectomized mice Since we are primarily concerned here with the effect of adrenal cortical secretions in the hypersensitive state I shall confine my discussion to allergic inflammation

All the animals used received a uniform challenging dose twenty one days after the same sensitizing doses of horse serum as were used in the previous studies on anaphylactic death The challenging dose of horse serum was 0 0005 ml horse serum per 20 gm mouse given in total volume of 0 25 ml saline In all experiments the challenging dose was injected three to four hours after adrenalectomy Five groups of control animals were used in the experiments a) nonsensitized intact animals b) sensitized intact animals c) sensitized adrenalectomized animals and d) sensitized adrenalectomized animals given 1 mg cortisone acetate by intraperitoneal injection two hours after adrenalectomy These four groups received no local challenging injection of horse serum A fifth control group was composed of intact sensitized animals receiving the challenging dose nineteen days after the second sensitizing dose This control group was introduced in order to study the inflammatory response in sensitized nonadrenalectomized animals At least seven animals were studied at each of the time intervals plotted in the Figures 19 and 20 At the end of this time interval the animals were sacrificed and the skin of the challenged area opened and subcutaneous connective tissue excised in thin sheets and spread one cell thick on glass slides Six or eight spreads were prepared from

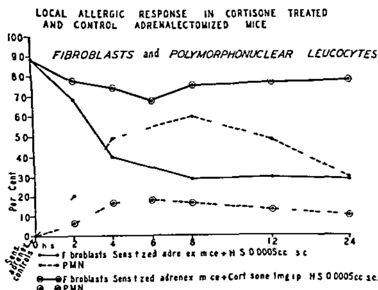


FIGURE 20

lactogenic substances on connective tissue, smooth muscle and endothelial cells Our evidence indicates that the histamine which is released in the allergic process probably does not come from the blood leucocytes a source which has been suggested by numerous authors (12)

The study of the quantitative relations between allergic reactions and adrenal cortical secretions would be entirely inadequate if the measurement of acute anaphylactic death were not supplemented by studies of less manifest and nonlethal sequels of hypersensitivity reactions We consider these studies as a particularly important phase of our investigations since these latter phenomena are thought to be responsible for the cardiovascular and collagen tissue pathology of the hypersensitive state (13) Accordingly the role of the secretion of the adrenal cortex in localized host reactions elicited by topically applied stimuli was evaluated by a quantitative method An attempt was made to secure information concerning the influence of secretions of the adrenal cortex on each of the various component events composing the inflammatory response Again, in order to eliminate the influence of endogenous hormones adrenalectomized animals were used The degree of involvement of the secretion of adrenal cortical hormone was then studied by comparing the topical inflammatory reaction of untreated adrenalectomized animals with that of adrenalectomized animals treated with cortisone acetate The effect of adrenal cortical hormones on the local inflammation produced by traumatic bacterial and allergic stimuli was evaluated by producing inflammation in the subcutaneous tissue of the abdominal region of intact and adrenal

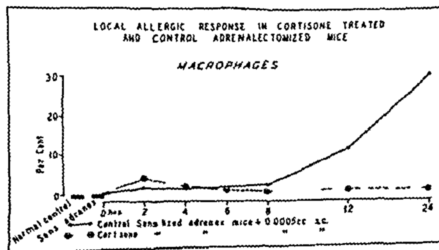


FIGURE 19

antiphlogistic action of cortisone provides an understanding of its ameliorative effect in various connective tissue diseases On the basis of evidence presented elsewhere (14) it is concluded that adrenal cortical secretions and exogenous cortisone inhibit allergic inflammation through an antiphlogistic action *sui generis* rather than by interfering with the antigen antibody union

It is likely that administration of large amounts of ACTH or cortisone can diminish the resistance of the organism by decreasing the inflammatory response Thus under certain conditions the pathological alterations produced by noxious stimuli such as bacterial toxins or viable bacteria may be enhanced by adrenal cortical hypersecretion This point may have considerable practical as well as theoretical significance with respect to the therapeutic uses of these hormones and the role they may play in the etiology of inflammatory diseases

To test whether cortisone exerts its antiphlogistic action locally or through a systemic mechanism it was injected into loose connective tissue of adrenalectomized mice simultaneously with a phlogogenous agent Histamine diphosphate (0 2 mg in 0 25 ml saline) was the standard inflaming stimulus The degree of inflammation was measured after six and twenty four hours by counting the number of damaged fibroblasts and nonautochthonous cells per mm ² of air dried stained spreads of inflamed tissue Amounts of cortisone from 0 5 mcgm to 20 mcgm prevented fibroblastic damage and significantly reduced invasion of PMNS and formation of macrophages (Figure 21)

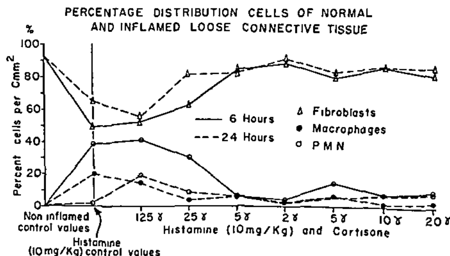


FIGURE 21

the area of inflammation in each of the animals Differential cell counts were then made of the spread and the percentage distribution (See Figures 19 and 20) of fibroblasts, polymorphonuclear leucocytes macrophages lymphocytes and eosinophils was determined

Sensitization alone did not produce any alterations in morphology or number of cells in the subcutaneous tissue with the exception of the eosinophils which were decreased in the connective tissue of the sensitized animals The local inflammatory response in challenged animals was qualitatively the same in intact and adrenalectomized mice The classical phenomena of fibroblast destruction and invasion of non autochthenous cells occurred However when cortisone was administered two hours before the challenging dose was given to adrenalectomized sensitized mice the inflammatory response was markedly decreased Destruction of fibroblasts was prevented and the invasion of polymorphonuclear leucocytes and macrophages was far less extensive The amount of cortisone given (1 mg per 20 gm mouse) did not completely suppress inflammation but did greatly diminish and shorten its extent Traumatically induced inflammation and inflammation produced by administration of pneumococci were similarly depressed

In summarizing this portion of the presentation I would like to quote from one of our publications (14) The procedure employed focuses the observation to the study of the *primary arena* of those exacerbations of physiologically fluctuating processes called inflammation and to their major performers the cellular elements of connective tissue And also the method allows the detailed study of changes in cellular morphology fluctuations in cell population and alterations in tissue structure in the sequence in which they characterize the destructive defensive and the reparative stages of inflammation We thus conclude that adrenal cortical secretions unquestionably have a marked *antiphlogistic* action on the inflammation produced by allergy as well as that which follows other phlogogenic stimuli This is indicated by *the facts that adrenalectomy noticeably increases the inflammatory re*sponse and that cortisone treatment profoundly decreases the cellular changes of inflammation The decrease in nonautochthonous cells could be due to the fact that there is a diminution in those substances which attract the leucocytes to the area of inflammation We believe that the absence of macrophages is due to the lack of migration of lymphocytes which are the precursors of the tissue phagocytes Our data does not refute the findings of Gordon and Katsh (15) who found adrenal cortical hormones essential for the maintenance of adequate phagocytic function of the fixed reticuloendothelial cells

The data just discussed support our earlier suggestion (4) that the

compared to the values of adrenalectomized nontreated control animals Histamine alone produced an eosinophilia

Doses of cortisone between 5 and 20 mcgm produced an eosinopenia when injected alone but not when given with the standard inflaming histamine dose Although these amounts of cortisone when combined with histamine did not produce an eosinopenia they did prevent the eosinophilia found to occur after administration of histamine alone

The data suggest that cortisone is utilized or inactivated while it is exerting its antiphlogistic activity since amounts of the hormone just sufficient to produce eosinopenia have less or no eosinopenic effect when simultaneously administered with an inflaming stimulus

Although our interpretation of this data must await chemical analyses for further confirmation the conclusions based on biological experimentation provide a strong case for the theory that cortisone is utilized while exerting its biological effects The fact that the varying degrees of inflammation and repair are dependent upon the ratio between the amount of inflaming stimulus and the adrenal cortical hormone supply opens up a broad new concept in the study of the etiology of connective tissue disease

DISCUSSION

Thorn What is the standard technique you use in preparing the animals for anaphylaxis? Are they in the fasting state?

Dougherty No At eight o clock in the morning they are adrenalectomized At ten we start the experiment Two hours are allowed to elapse between adrenalectomy and the start of the experiments Other experiments have indicated that the mice are as sensitive immediately after adrenalectomy as they are after a lapse of two hours

Thorn I agree that there is antagonism between DCA and cortisone but it is important to point out that the dose of desoxycorticosterone used in the mice experiments is the same as for a human patient with Addison s disease Therefore the possibility of toxic action of the steroids in the mice must be considered before one can speak specifically of competitive reactions The dose of desoxycorticosterone is much larger than the corresponding doses of cortisone

Dougherty We have some evidence that at very low doses i e doses of cortisone just on the borderline of preventing inflammation cortisone can be antagonized mole for mole by DCA However larger doses of cortisone and DCA do not show this mole for mole inhibition

Administration of 0 25 mcgm did not inhibit significantly the initial alterations of inflammation normally seen after six hours but did prevent the formation of macrophages usually present at twenty four hours A lower dose (0 125 mcgm) had no effect on the course of the inflammatory phenomena The data suggest that the antiphlogistic action of cortisone follows a graded dose response curve and that there is a direct relation between the degree of action of a phlogogenic agent and the amount of hormone required to prevent its inflammatory effect

In order to determine whether cortisone prevents inflammation through local or through systemic action blood eosinophil counts were performed on the adrenalectomized animals given histamine either alone or combined with the various cortisone doses employed Results of these experiments are reported in Figure 22 Adrenalectomy alone produces an eosinopenia within six hours after operation The deviations in eosinophil counts of the experimental groups must thus be

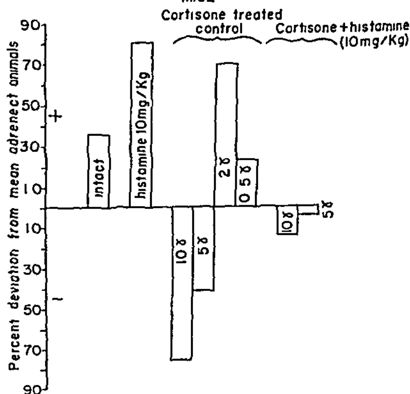


FIGURE 22

carboxymethylcellulose turned out to be chiefly responsible for these changes Tween 80 was weakly effective Other colloids than those used in Cortone (Merck) have been studied such as pectin polyvinyl alcohol and methylcellulose Of these only pectin produced similar azurophilic inclusions

The inclusions are strictly topical when suspending agent without the hormone is given to adrenalectomized animals but appear ubiquitously *in distant connective tissue areas when cortisone is included* Tween 80 and carboxymethylcellulose are both inflaming agents and the macrophages attracted to the inflamed area may also contain the inclusions Endothelial and perithelial cells are similarly affected No micellophagosis was displayed by fixed reticuloendothelial cells

Repeated injections of such inclusion producing substances increased the number of affected cells and their number of inclusions These observations suggest that although affected cells are not severely damaged the presence of the large amount of material within their cytoplasm must interfere with cellular function The changes in the staining reaction of amorphous ground substance and the localized edema suggest alterations in capillary permeability The presence of inclusions in endothelial cells may be partially responsible for these alterations Recently Menkin described similar changes in capillary permeability produced by cortisone suspending agents (18)

Since micellophagosis inducing substances were found to be intensely phlogogenic it is likely that much of the cortisone in the preparations given may be utilized in counteracting the inflammation produced by the suspending agent and not be available for other purposes

Thorn The sensitivity which we have observed in the use of intramuscular cortisone suspensions has occurred in patients with Addison s disease who have been given relatively small doses of cortisone i e 12 5 to 25 mg daily These patients are often hypersensitive to medication anyway

Astwood Have you tried staining the material itself?

Dougherty Yes Our results are not conclusive When spread and dried on a slide the material stains a pink color It is apparent that the azure in the polychrome dye is the effective staining agent However no granules are stained it is a diffuse color Therefore at present we conclude that further investigation is necessary to determine whether the inclusions represent the actual detergent itself or represent changes in the cytoplasm It is interesting to speculate as to the site of action of the C 11 oxysteroids Several possibilities exist but we believe that the C 11 oxysteroids inhibit the union of antigen antibody and that this is their site of action

White If this is the site of action of the C 11 oxysteroids how do

according to our preliminary experiments

Thorn I should guess that if this is a competitive action it could be shown with one twenty fifth of that dose rather than with a full milli gram

Selye May I ask if you have ever found basophilic intranuclear inclusions in the endothelial cells?

Dougherty I have not

Selye In some animals ACTH and LAP were given simultaneously and nuclear inclusion bodies were found in the kidney These may become very large They look like giant nucleoli or like virus bodies

White In what portion of the kidney?

Selye In the tubules, and also in the glomeruli In one particular experiment out of ten animals so treated nine had inclusion bodies

White Do you see this with continued ACTH alone?

Selye No, I have never seen it, nor did I find it after LAP in any organ other than the kidney

May I ask what are the very large eosinophilic cells in Figures 15 and 16?

Dougherty They are fibroblasts which have inclusions produced by the cortisone suspending agent At first we thought they were produced by cortisone (16) Immediately following administration of cortisone histological examination showed changes in amorphous ground substance which became edematous and changed in its staining capacity Within four hours (but not before) the fibroblasts in the area began to accumulate large acidophilic cytoplasmic inclusions of irregular size and shape (Figures 15 and 16) The number of the inclusions varied from cell to cell from only a few up to a hundred or more The size of the inclusions varied from 0 5 to 2 microns The changes do not seem to be degenerative and the nuclei remain quite normal Since the inclusions are found in fibroblasts which ordinarily are not actively phagocytic in their fixed position and they do not contain cytoplasmic vacuoles this phenomenon does not appear to be typical phagocytosis Consequently we have given a name to this phenomenon— micellophagosis

Recent investigations (17) indicate that 40 per cent of the fibroblasts are affected within the area of subcutaneous injection in adrenalecto mized mice This effect appears at four hours and is maximal twenty four hours after injection The inclusions disappear very slowly and cells may contain them for a matter of ten days to two weeks after a single injection of as little as is present in 5 mg of suspended cortisone acetate (We do not know the exact concentration of suspending agents used in preparation of Cortone)

Among the constituents of cortisone free suspending agent sodium

mation may be absent so the individual does not feel sick

Astwood I am not sure that that necessarily follows

Dougherty That I admit does not necessarily follow In other words we don t know that phagocytosis is important but I suspect it is

Bauer Dr Maxwell Finland has treated several patients with lobar pneumonia with ACTH They became afebrile promptly and soon experienced a sense of well being The clinical course of the pneumonia as judged by physical and X ray examination was the same as that of untreated disease

White The organism persisted?

Bauer The organism persisted in the sputum

Dougherty If they recovered finally I do not know the meaning of phagocytosis I would suspect that the experience of a great many years teaches us that phagocytosis is fairly important

Astwood That is the point That is what was taught us all these years but these recent experiments indicate perhaps that this is not so

Dougherty I can only say that further study is necessary

Thorn We know that cortisone administration may aggravate pulmonary tuberculosis under certain circumstances It appears that in the case of bacterial disease which depends upon the body s capacity to wall off an inflammatory reaction it may permit extension of the organisms

Dougherty I think there will be evidence on that I would rather not take up the question which Dr Astwood brings up at the present time until we have experimental evidence I think Finland s work can be explained and still keep phagocytes in the picture I agree with Hans Zinsser s results that antibodies are important but we still need the phagocytic cells

Fremont Smith Will you explain why invasion of phagocytes does not take place how the invasion from the capillaries is inhibited?

Dougherty We do not know why invasion of leucocytes at the site of inflammation is inhibited by cortisone We think that the leucocytes do not migrate because either the substances which attract them to the area are not present or are incapable of acting on normal endothelium so that margination of leucocytes does not occur The former explanation appears to be more plausible

Fremont Smith The endothelial cells do not become sticky?

Dougherty Correct At least according to our preliminary experiments the endothelium is not sticky at the site of inflammation in animals treated with large doses of cortisone

Fremont Smith Did not Chambers and Zweifach have some experiments on that very point?

Dougherty I do not know

you account for the fact that the normal animal with similar histological changes does not die?

Dougherty The reason I think, is this The endothelial changes are the first gross alterations produced by the antigen antibody union They may be most important as far as long continued exposure to allergens is concerned but are not immediately responsible for acute anaphylactic death which results from smooth muscle contraction Thus cortisone may not be capable of inhibiting the first explosive effect of antigen antibody union but may diminish the concentration of those substances such as histamine which produce bronchiolar smooth muscle contraction which results in death

Thorn I think all of your points have been substantiated by Dr C B Favour s work in our department

Dougherty Favour s work is of the greatest importance in the understanding of the etiologic mechanisms of hypersensitivity and should be understood by all pathologists The reason for this strong statement is that he has shown that there is a mobile source of antibody containing cells which may upon coming in contact with the proper antigen produce inflammation in all tissues

Loewi Do the macrophages come from the capillaries? If not from where?

Dougherty In answering Dr Loewi s question I should like to state that the macrophages come from the lymphocytes which migrate through the capillaries at the site of inflammation The main line of defense of the organism is the macrophage response of the loose connective tissue which we can almost completely eliminate by overdosage with cortisone This we have now found is not only true with allergic inflammation but is true as well with histamine and with living pneumococci

Loewi Do the macrophages directly act in the tissue or on the capillaries?

Dougherty We do not know as yet In any event there are less macrophages in inflamed areas of animals given large doses of cortisone I should think there might be some danger in treating an individual with an acute infectious disease with cortisone in large doses because the phagocytic response even to the pneumococcus can be almost completely eliminated

Thorn The difficulty lies in the fact that one does not observe the manifestations of disease from pneumococcus infection (signs and symptoms of pneumonia) until the hormonal treatment has been discontinued

Dougherty You simply do not have any phagocytes to take up the organisms and the organisms will continue to grow Of course inflam

Dougherty With respect to phagocytic function the two groups of cells have differences and similarities There are few macrophages in normal loose connective tissue The histocytes found in inflamed connective tissue are mainly derived from invading blood lymphocytes The fixed reticuloendothelial cells are capable of phagocytosis at all times Thus in the first instance the macrophage precursors must be attracted to the inflamed area—a phenomenon which we have shown to be regulated by adrenal cortical secretions The maintenance of adequate phagocytic function itself for either group of macrophages may also require adrenal cortical hormones This is indicated by the work of Gordon and Katsh referred to previously (15)

White Is it possible that the activation of such a system of macrophages might function in a compensatory manner in an infection treated with adrenal cortical extract?

Dougherty Definitely The site of inflammation virulence of bacteria amount of fixed reticuloendothelium amount of circulating antibodies whether antibody is already formed at the start of hormone treatment regulation of fixed macrophagic activity by adrenal cortical hormones the extent of elimination of bacteria at the time of hormone treatment the presence of allergy and a host of other variables enter the picture and should be analyzed before adrenal cortical hormone therapy can be truly evaluated

Thorn Have you ever considered how remarkably well the Addisonian when his Addison s disease is due to tuberculosis walls off tuberculosis and lives with the disease for an indefinite period This is in contrast to the frequently stated idea that Addisonian patients are more susceptible to infection than others which I do not believe to be true I do believe that with a given infection their capacity to resist the stress of infection is inferior to that of other patients but their capacity to wall off infection provided they can survive may actually be better than normal

Dougherty The Addisonian may be hypersusceptible to the toxic products released by bacteria tissue damage and allergy but his capacity to meet invasion by inflammation and wall it off etc may be increased as is the case with adrenalectomized mice (14)

Thorn If you could demonstrate diminished production in the hemolytic anemia system it would be a nice study

Dougherty The antigen antibody union and therefore release of anaphylotoxins is not inhibited by cortisone in the hemolytic anemia system *in vitro* (21) In erythroblastosis Dr Holmstrom* in preliminary studies found that ACTH does not appear consistently to

* Unpublished data

Fremont Smith I think it would be worth while to ask Zweifach I have a vague memory that in his early work on shock on the mouse mesoappendix and possibly on the frog, he had some adrenal cortical extract which blocked the stickiness

Dougherty We have designed a method similar to that used by Zweifach in which mouse mesentery can be used Although we have not used this method sufficiently to acquire a great deal of confidence we believe that stickiness is prevented in the mouse by cortisone

Thorn In 1936 we carried on some experiments with McAllister and Gregerson in Baltimore They had a beautifully standardized dog in which a constant decrease in plasma volume could be obtained with a standard inhalation anesthesia Using this technique it was shown that the administration of a potent adrenal cortical extract completely prevented the decrease in plasma volume which had previously been shown to occur in the same animal for the same period of anesthesia We felt that this was an indication of a primary vascular effect of adrenal cortical hormone

Pincus Is there any lymphatic block at all?

Dougherty I do not know One might be able to study this in the mouse ear So far we have been mainly interested in the cellular aspects of inflammation

Pincus In dealing with the inflammatory process if I remember correctly Menkin states that this is one of the primary leukotaxine effects

Dougherty I believe so He also showed that capillary permeability as far as penetration of colloids is concerned appears before any distinct morphological change in the endothelium takes place He demonstrated that adrenal cortical extract could inhibit the diffusion of trypan blue through capillary walls (19)

Ralli Do you think that this bears some relation to the fact that with ACTH treatment skin grafts take on burned areas and are not sloughed off or phagocytized?

Dougherty That is an excellent suggestion Leo Loeb (20) showed many years ago that the take of transplants is affected by the degree of inflammation they induce Also he called attention to the fact that lymphocytes are the most reactive cells to transplants and are responsible for destroying the foreign tissue Thus the decrease in inflammatory response and diminution of lymphatic tissue produced by hormones of the adrenal cortex may promote takes of homologous or even heterologous tissue

White Would you distinguish between this process of appearance of macrophages in tissues as contrasted with the activity of fixed macrophages in relation to adrenal cortical activity?

ml locally has given the same effect as 1 ml systemically in the mouse

Pincus As I recall Rich s work the antihistamines were not particu larly effective in preventing the lesions that he observed and yet you indicate histamine as a very important product of the antigen antibody relation

Dougherty As far as anaphylactic shock is concerned Dragstedt (22) lists at least several substances which are released—histamine heparin choline and adenosine etc

White Choline or acetylcholine?

Dougherty Choline and acetylcholine too plus other substances Dragstedt emphasizes that we must not put sole emphasis on Mine mine mine you decarboxylated son of imidazole histidine Thus the ineffectiveness of antihistaminics cannot even be inferred to preclude the theory of connective tissue disease as proposed by Rich Apparently antihistamines may block certain actions of histamine at some sites but not in all tissues In fact certain toxic actions of histamine may be enhanced by antihistaminic substances (23)

Pincus What dissuaded you from thinking the interference might be with the antigen antibody reaction if antihistamines do not affect this?

Dougherty Apparently antihistamines do not eliminate the antigen antibody union or destroy histamine but block the action of histamine released by allergic phenomena (23) Since cortisone does not inhibit the antigen antibody union *in vitro* or the endothelial damage *in vivo* it appears that it does not act at this level or as a histamine blocking agent either This hormone is more likely concerned with the *in vivo* destruction or elimination of histamine released by the antigen antibody union

Thorn Favour in our department has carried out some interesting experiments on tuberculosis He sensitizes animals to tuberculin over a prolonged period of time Then if one injects a small amount of tuber culin into such an animal one observes the development of a necrotic area If the animals after they have been sensitized are treated with an adequate dose of ACTH there will be no over all reaction to tuber culin but the damage of the local cells by the tuberculin has been unaffected

Selye I wonder whether Dr Dougherty would tell us a little more about his ideas on the competition which I think is most interesting and important

In our experiments on topical irritation arthritis and also in our work on the anaphylactoid reaction to egg white we noted—in con firmation of what you just said—that after adrenalectomy these condi tions are aggravated by DCA and ameliorated by cortisone In this

decrease antibody of the mother prior to parturition In the hemolytic anemia of the newborn it seems to have little effect on the antigen antibody union although it apparently aids in maintaining the infant s hemoglobin

Thorn Nor does it block histamine action per se

Dougherty Apparently not It does not block the action of histamine *in vitro* nor does it inhibit the Schultz Dale phenomenon according to Loewe *

Thorn It is possible to go one step further on the basis of what you have said and on the basis of our comparative studies which I shall show tomorrow of injected cortisone versus orally administered cortisone There is a very great probability that injected cortisone with a detergent or without it may permit the local destruction of cortisone before it becomes available to the organism We have no evidence that a milligram of cortisone injected intramuscularly has the same level of activity as a similar quantity of orally administered hormone in terms of eosinopenic response It would not surprise me at all to find that an appreciable quantity of hormone was utilized locally in the tissue particularly if inflammation was present or if you give it with the detergent and the detergent in turn stimulates a reaction

In the light of this hypothesis we have had a single interesting experience in a patient with widespread rheumatoid arthritis but with particular involvement of one knee in whom we administered into the knee joint 10 mg of compound F provided by the Upjohn Company We were hoping of course to be able to demonstrate a local effect on the knee joint without necessarily showing improvement throughout the rest of the body The interesting fact is that within two or three hours after the injection into the knee this patient who had not been out of bed in almost a year was walking around I have never been able to explain why 10 mg of hormone were so effective throughout the body unless she already had a normal level of hormone in her blood but did not have a high enough level locally in her inflamed tissue

Dougherty Your experiments emphasize the need for further knowledge concerning utilization and whether or not a steady state exists between the blood and tissue levels of the hormone It may be that this latter point will give us a clue for more rational therapy and further understanding of connective tissue disease

Opsahl With reference to Dr Thorn s remarks the smallest amount of compound F we have used locally is 0 05 mg which has given remarkable results in terms of inhibition of hyaluronidase enhanced spreading In terms of adrenal cortical extract administration 0 025

Unpublished data

the difference lies—when we can identify this difference we are then on the road to progress For example I think it was Lee who originally reported that histamine caused a rise in spinal fluid pressure and later reported that histamine lowered spinal fluid pressure Finally it was found that the differences were due to the anesthesia used—without anesthesia or with ether anesthesia histamine caused a rise with barbiturates histamine lowered the spinal fluid pressure This illustrates the importance of ascertaining the *differences* in the *same* experiments

Selye Let us do this How much DCA did you inject?

Sayers We implanted twelve pellets in animals which were unilaterally nephrectomized They had 0 9 per cent sodium chloride solution to drink They were on a diet which was composed of 94 per cent calf meal 3 per cent wheat germ and 3 per cent dried brewers yeast

Fremont Smith What animal was used?

Sayers The Sprague Dawley rat The cortisone dosage was 2 mg per day We also used 0 1 mg per day but this dose was entirely inadequate in inhibiting the development of lesions Two milligrams of cortisone per day started immediately following DCA implantation and continued until sacrifice markedly inhibited the development of lesions Adrenocorticotropic hormone in doses of 1 10 and 50 mcgm per day did not protect against DCA ACTH in doses of 0 5 or 3 mg per day protected adequately

Fremont Smith What age were the rats?

Sayers The animals weighed 275 to 350 gm The duration of the experiment was forty days

Long Five milligrams each twelve pellets?

Sayers The pellets were 15 mg About 0 2 mg of DCA is absorbed from each pellet per day

Selye We gave 2 5 mg of DCA per day in animals on a Purina diet with 1 per cent sodium chloride I wonder how large your animals were

Sayers Male rats weighing 275 to 350 gm

Selye I worked with considerably smaller animals usually under 100 gm and rarely above 130 gm In many experiments during the last few years we never used rats larger than this for such experiments This may be an important factor The dosage level seems to be quite comparable since I give 2 5 mg per day in the form of a microcrystal suspension

Fremont Smith How about the diet?

Selye Purina Fox Chow My animals were unilaterally nephrectomized and given excess sodium chloride just as in Dr Sayers s experiments

Fremont Smith Is there any possibility that the preparations injected

respect there appears to be some antagonism between these two steroids We ascribed this to a possible competition between the two hormones similar to the competition for the substrate which occurs in enzyme reactions (24 25)

Such inhibition does not occur at all target organs however For instance, I have never been able to inhibit the thymolytic effect of cortisone with DCA and of course the life maintaining action of the two steroids is additive Furthermore as I have said previously the renal damage caused by DCA is further aggravated by cortisone I am speaking of course of histologic lesions in the kidney It appears to me that if there is some such mechanism of competition for a substrate, this would have to be visualized as being limited to certain target organs only

Thorn The kidney is not a good example because in this organ the action of these steroids will be competitive There is no evidence that cortisone or ACTH effectively modify the inflammatory lesions of the kidney and cortisone has failed to cause a constant or striking improvement in acute subacute or chronic nephritis The inflammatory lesions in the liver and kidney appear to respond quite differently to ACTH and cortisone and to the reticuloendothelial tissue

Sayers Dr Woodbury Dr Rosenberg and I have been able to antagonize the action of DCA in inducing pathological changes in the kidney Both cortisone and ACTH if given at the appropriate dose level will inhibit the development of pathological changes in an animal treated with DCA

Thorn Exactly what do they antagonize?

Sayers *Pathological changes in the kidney (glomerular and inter*stitial nephritis) which occur quite frequently in rats given DCA and on a high sodium intake the changes were noted on histological examination

Selye Under the conditions in which we have performed such experiments we found precisely the opposite (26) The kidneys of *animals simultaneously treated with DCA and cortisone were consider*ably more damaged than those of rats receiving one of the two steroids alone

Long May I ask if anybody has done any actual measurements of renal function?

Dougherty Have the functional changes been studied in animals exhibiting histological changes induced by DCA?

Fremont Smith I am always intrigued when two people report that they have done the same experiments and obtained different results —it is not quite conceivable that exactly the same experiments would produce different results! The important thing is to determine where

perhaps an extension of the problems that were discussed by Dr Dougherty the subject of Hyaluronidase and the Adrenal Cortical Hormones which will be opened by Dr Opsahl

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were differently prepared?

Selye Yes Dr Sayers used pellets of DCA and I used microcrystal saline suspensions

Fremont Smith Suspended in what?

Selye In Merck s suspension agent

Fremont Smith Did you have any control on the suspension?

Selye We had microcrystals of cholesterol

White We have recent evidence that the age of the animal may have been an important influence Dr Adams who was with us last year and has migrated to Salt Lake City did a series of experiments in which the difference in age of mice of eight weeks as compared to ten weeks was the important factor determining whether or not the animal s liver became fatty on fasting Animals from six and eight weeks did have fatty livers under such experimental conditions Animals ten weeks of age did not show fatty livers on fasting

Ingle Female rats?

Selye Yes

Fremont Smith A difference in sex and age could make a difference

Thorn Age is most important

Dougherty Since suspending agents are phlogogenic cause fibro blastic and endothelial alterations and are possibly antigenic their use should be carefully controlled in experiments in which connective tissue lesions are produced by hormone treatment

Thorn They both used cortisone?

Dougherty But Dr Selye suspended his

Sayers Because of the influence of Dr Dougherty we did *not* use cortisone suspended in detergent We gave cortisone acetate dissolved in ethanol (10 per cent of the total volume) and suspended in 0 5 per cent sodium-chloride solution (90 per cent of the total volume)

Selye While I used a suspending agent for the DCA and cortisone I did not use it for the ACTH and obtained the same effect there

Fremont Smith I hope very much that by the time of the next Con ference we will have a report on the further specification of this differ ence because I think it illustrates so clearly how differing results are obtained by so called identical experiments I hope that each or both of you will repeat the experiment using the controls of the other A report of this kind should be most interesting

Selye Certainly I would be glad to do it with adults I think the age factor is very likely to be important

Thorn It is in man

Ingle Did you use litter mates?

Selye We did not use litter mate rats

Long We will proceed to our next topic In a certain sense it is

HYALURONIDASE AND THE ADRENAL CORTICAL HORMONES[*]

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In 1942 Duran Reynals in a comprehensive review on the subject of tissue permeability and the spreading factors in infection pointed out that among the many factors conditioning the permeability of the ground substance of the mesenchyme hormones play an important part The influence of a variety of hormones on the spreading effects of testicular extracts had been studied but no definite conclusions were justified by the results obtained In view of the numerous controversial papers relative to the effects of the adrenal cortical hormones on capillary permeability it is surprising that relatively little attention was given to the influence of these hormones on hyaluronidase activity Valy Menkin had shown that extracts of the adrenal cortex markedly inhibit the enhancing effect of leukotaxine on capillary permeability There was some indication from Menkin s work that this effect was duplicated in experiments with testicular extracts however he did not investigate these points in any detail It was of interest to us to investigate the interrelationships that might exist between hormones of the adrenal cortex the enzyme hyaluronidase and its substrate hyaluronic acid as well as the general problem of the permeability of the ground substance of the mesenchyme As the studies progressed several problems presented themselves a) which fractions of the adrenal cortex were responsible for the results obtained b) what was the mechanism of the action seen and c) in what way was the hyaluronic acid metabolism involved in the arthritides and in other collagen diseases?

It might be pertinent to digress for a moment with regard to the general structure of connective tissue You will recall as Meyer has pointed out in numerous papers dealing with the subject of the mucoproteins and the mucopolysaccharides that the cement substances together with the fibrous elements are the two chief components of connective tissue The cement substances are compounds or complexes

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[*] Som of th m t l u d n this pape h l dy been publ hed d m y be found in the fi t f efe l t d page 161

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injections on mice were made under light ether anesthesia for while studies employing just killed animals proved satisfactory for pre liminary experiments they offered definite limitations as to procedures and interpretations of results After varying time intervals the animals were killed skinned and the skins fastened to mouse boards Tracings from the under surface of the dry skins were made on small cards These tracings were photographically enlarged and planimeter meas urements were then made The hyaluronidases used were standardized preparations prepared from testicular tissue and supplied by Dr Joseph Seifter of Wyeth Inc The dilution of enzyme used in most of these studies was 1 100 or 1 1000 and no significant differences were noted when varying diluents were tested It was experimentally found that mixing the India ink and enzyme solution or control saline immediately before injection was satisfactory The experimentally determined maxi mal volume that can be satisfactorily injected intradermally in the mouse is 0 05 ml

An idea of the appearance of these skins after injection of India ink and hyaluronidase or saline under the various experimental conditions is shown in Figure 23 The blanched areas seen are merely due to the



Normal



Adrenalectomized



Normal + ACE



Adrenalectomized + ACE

FIGURE 23 * Photograph illustrating changes in dermal spread of India ink with or without hyaluronidase as influenced by the intra peritoneal injection of ACE in normal and in adrenalectomized mice

* Reprinted from Yale J Biol & Med 21 255 (1949)

of protein with highly polymerized mucopolysaccaride acids and while the chemical nature of these proteins is unknown, of the mucopolysaccharides which are more or less loosely bound to the protein four have been identified as components of the cement substances a) hyaluronic acid, b) hyaluronosulfuric acid, c) chondroitin sulfuric acid and, d) the sulfuric acid ester which occurs in amyloid tissue Hyaluronic acid is a polymer of a disaccharide composed of N acetyl glucosamine and glucuronic acid Its exact structure is unknown and its molecular weight varies with its source Hyaluronic acid is depolymerized and hydrolyzed by specific enzymes called hyaluronidases which occur in microorganisms such as streptococci staphlococci, pneumococci, and certain gas gangrene producing organisms Hyaluronidase is found in the spermatazoa of animals in snake venoms and in the leech In the human body the greatest store seems to be the skin where however it appears to be largely in an inactive form This latter point has been the subject of much controversy It is interesting that synovial fluids, vitreous humor and tumor fluids contain only hyaluronic acid Umbilical cord and skin contain hyaluronic acid and chondroitin sulfuric acid in about equal concentrations while cartilage contains chondroitin sulfuric acid only It is believed that chondroitin sulfates are hydrolyzed by some hyaluronidases I believe this introduction provides sufficient background for consideration of our studies

Time does not permit giving other than representative experiments covering a few phases of our investigations nor can I mention the innumerable experiments that resulted in certain standard procedures now commonly employed in these studies In the animal studies for the most part mice of the inbred CBA strain were used however experiments were also carried out using rabbits as larger test animals We breed and raise our own colony of mice under rigorously standardized conditions and to date we have used approximately 10,000 in these experiments

As it has been well established that a variety of physiological factors influence the pituitary adrenal cortical system it was first necessary to devise assay procedures that were as free as possible from stress factors for the detection and measurement of hyaluronidase activity Male and female mice were used at ten to twelve weeks of age The general approach was to inject intraperitoneally intramuscularly or subcutaneously varying amounts of adrenal extract (Wilson s) followed at time intervals of one to six hours by the intradermal injection of small amounts of hyaluronidase or saline using India ink as the indicator into bilateral comparable areas of the shaved flanks and to determine the degree of spreading under various experimental conditions Control experiments were done in all cases For the most part intradermal

Effects of injection of adrenal cortical hormone and of adrenalectomy on the permeability of the ground substance

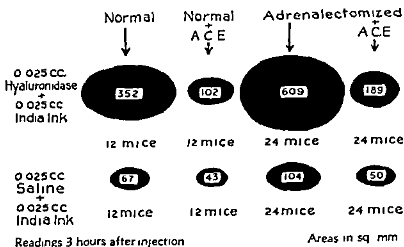


FIGURE 24*

because the areas of spreading were decreased below control values

Innumerable studies were carried out using different amounts of adrenal cortical extract and hyaluronidase The effects of varying the time intervals of injections were also studied as well as a thorough investigation of any contributing roles that might be played by the variables in the method

Similarly a complete series of investigations using lipoadrenal extract were carried out and an increased effectiveness in smaller doses as compared with ACE was noted

In conjunction with other phases of these investigations we carried out a complete series of studies with ACTH Injection of amounts varying from 0 5 to 50 mg intraperitoneally intramuscularly or subcutaneously produced similar marked degrees of inhibition in the normal or castrate animals and were without effect in the adrenalectomized animal Locally ACTH was without effect

It is interesting that DCA had no effect on the spreading caused by hyaluronidase—this finding paralleled the observation that DCA in contrast to active extracts of the adrenal cortex is not effective in causing a dissolution of lymphocytes in the animal body

The studies just mentioned demonstrated conclusively that some factor elaborated by the adrenal cortex was a potent inhibitor of the spreading reaction Whether this effect was one of inhibition on enzyme or substrate was not known other than that there appeared to be an effect on both the greater inhibition being observed in the presence

* R p t d t Y l J B l & M d 21 255 (1949)

shaving of the other surface of the skin in order to make more accurate injections

When adrenal cortical extract was injected intraperitoneally subcutaneously, or intramuscularly followed at time intervals of one to six hours by the intradermal injection of saline or enzyme solution with India ink the spreading effect as induced by hyaluronidase was found to be markedly inhibited This restricting effect was also seen to a lesser degree on the control saline injection indicating the possibility of action on substrate as well as upon enzyme Adrenalectomy resulted in a marked enhancement of the spreading reaction in living animals However the effect of adrenalectomy could not be obtained in animals killed just prior to the intradermal injection of India ink and hyaluronidase The effect of adrenalectomy can be entirely counteracted by the intraperitoneal injection of adrenal extract As controls for adrenalectomies sham operations were routinely carried out and all animals were exposed to the same degree of ether anesthesia The experiments with adrenalectomized animals indicate that depletion of the adrenal factor concerned with the spreading reaction is rapid This conclusion can be drawn since very large areas of spread were entirely comparable to those observed six days following adrenalectomy Because of the time factor many experiments cannot be detailed although mention of a few points should be made The obvious question of the effect of the Merthiolate the preservative for the ACE was investigated and was found to play no part in the inhibiting effect either upon systemic or local injection leaving little doubt that the effective agent was actually derived from the adrenal gland

Figure 24 illustrates diagrammatically a representative experiment showing the changes in spreading that are brought about by the adrenal The areas shown represent the average area found after the injection of India ink and hyaluronidase or saline into intact or adrenalectomized mice The numbers written in the darkened areas give the average area in square millimeters The numbers beneath the blackened areas represent the number of animals employed in each group In each case the hyaluronidase had its characteristic effect in enhancing the area of spreading of India ink as compared to that found when saline was injected instead of the enzyme Adrenal cortical extract decreased spreading in both saline controls as well as in the enzyme infected mice Removal of the adrenals one or six days prior to injecting enzyme or saline solution resulted in the relatively tremendous increase in spreading as is illustrated The data in the last diagram show quite clearly that the injection of adrenal cortical extract into adrenalectomized mice entirely counteracted the effect of the adrenalectomy In fact, there was some evidence of excess hormonal action

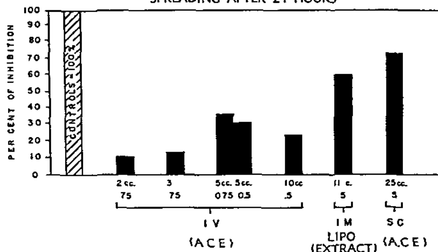


FIGURE 25 Photograph illustrating changes in the dermal spread of India ink with or without hyaluronidase as influenced by the systemic administration of ACE twenty four hour spreading Left side is control Right side is test

of the added enzyme While it was not possible to conclude that this represented a direct inhibition of the enzyme hyaluronidase in conjunction with the evidence presented by Meyer it is possible to assume that hyaluronidase is normally present in the skin and that the adrenal effects observed in the saline treated animals may have been due to an influence on the hyaluronidase enzyme system

Dr White mentioned at last year s Conference that during the course of these studies a curious observation was made upon the effect of environmental temperature and humidity on the influence of adrenal cortical hormones and other agents upon the spreading effect of hyaluronidase During a portion of the summer of 1947, there was a period when the air-conditioning apparatus in the mouse house was inoperative and the mice were exposed to elevated environmental temperatures Contrary to earlier results it was observed in the control experiments that the areas of spreading of India ink both with and without hyaluronidase were smaller than had been obtained previously and that in addition no further inhibiting effect of adrenal cortical extract on the spreading of India ink was found This was surprising in view of the fact that previously there had been excellent reproducibility of results with little variation of values between individual animals and those of experiments carried out at widely varying intervals We of course repeated the experiments many times under similar conditions at that time and obtained entirely comparable results We questioned the stability of the hyaluronidase preparations and adrenal cortical extracts and studied the effects of variation of the diluents but the results were the same One month later when temperature and humidity controls were reestablished the previously noted ranges of values for areas of spreading in normal animals were again demonstrable and in experiments involving the use of adrenal cortical extract the inhibiting effect was again obtained During the next summer in 1948 groups of animals were brought from the air-conditioned mouse house into the laboratory and kept under the existing high temperature and increased humidity conditions and results comparable to those found during the previous summer were again demonstrable The points to be stressed in these observations were that under conditions of increased environmental temperature and humidity the areas of intradermal spreading of India ink with or without hyaluronidase were markedly reduced and appeared to be comparable to a degree to that resulting from the injection of adrenal cortical extracts and that the superimposed injection of such extracts appeared to give no further inhibition We reported these findings as suggestive that increased environmental temperatures and humidity may play a role in stimulating the adrenals and thus cause a decrease of the permeability of

THE INHIBITORY EFFECT OF SYSTEMICALLY ADMINISTERED A.C.E. OR LIPOADRENAL EXTRACT OF INDIA INK ENZYME SPREADING AFTER 24 HOURS



PER CENT INHIBITION = $\frac{\text{CONTROL AREA} - \text{TEST AREA}}{\text{CONTROL AREA}}$

Average Values are given A control was done in conjunction with each test study

FIGURE 26

effect had been dissipated and the question arose as to whether or not a greater inhibition might not have been obtained if a shortened spreading time had been allowed The extreme degrees of inhibition that can be obtained by massive doses of adrenal hormones are clearly demonstrated in the response to large doses of either lipoadrenal extract given subcutaneously or adrenal cortical extract (aqueous) given intramuscularly The values given are from measurements made after a twenty four hour period but in spite of this long postinjective time areas of spread were inhibited 61 and 73 per cent respectively

These intravenous experiments were repeated and Figure 27 shows that with the larger doses of adrenal cortical extract administered intravenously the inhibition of hyaluronidase enhanced spread diminishes markedly after the first hour The rapid excretion of adrenal steroids as pointed out by Venning offers a logical explanation for the diminished effectiveness of ACE noted here It is of particular significance that in spite of the inhibition of hyaluronidase by doses of adrenal extract in the range of 2 or 3 ml maximal effects probably require dosages exceeding those employed in these intravenous studies Loss of effectiveness within a short time after injection accounts to some extent for the large doses required The massive doses of adrenal hormones necessary for the establishment of a maximal response

the ground substance

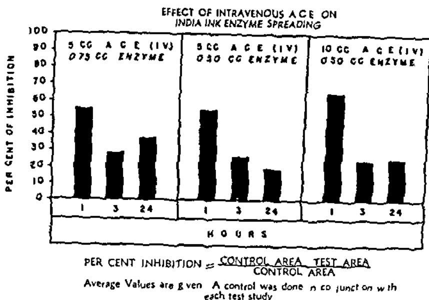
Having established the adrenal hyaluronidase relationship in mice we turned to the rabbit as a second test animal our interest being to investigate some of the experimental variables in this field and thereby establish a basis for further experimental procedures Male New Zealand white rabbits were used and since these animals are unusually susceptible to stress relatively tamed rabbits were prepared for later study and no anesthesia was used An important observation is the marked difference in degree of spreading of India ink with hyaluronidase when injections are made into various areas of the flank or abdomen of individual rabbits Control tests in our studies indicated that this was the case and controls were always done in conjunction with the experimental testing comparable bilateral areas were invariably used Figure 25 shows the appearance of one of these rabbit skins It was determined experimentally that there is a variable degree of continued spreading both in saline and in enzyme treated areas up to twenty four hours Control injections of saline or enzyme solution with India ink were made on one side of the shaved rabbit At the end of the twenty four hour interval following the injection of enzyme or saline with India ink the adrenal extract was injected intravenously Following the adrenal extract injection a three hour time interval was arbitrarily selected for the effects of the injected extract to become manifest At the end of this time interval similar amounts of saline or hyaluronidase with India ink were made into comparable bilateral areas on the opposite side At the end of the twenty four hour test interval the rabbits were killed and skinned and measurements made as previously described In certain groups of experiments measurements from the outer surface of the skin were made after one three and twenty four hours You can see the inhibiting effect on hyaluronidase enhanced spreading as produced by the intravenous injection of adrenal cortical extract

In Figure 26 are shown the results of intramuscular and subcutaneous injection as well as intravenous injection of certain of these extracts This figure summarizes diagrammatically some of the inhibitory effects produced by the administration of ACE or lipoadrenal extract with respect to dosages routes of administration and time intervals employed The values are averages of experiments of groups of animals Inhibition of the spreading which resulted from the intravenous administration of ACE in doses of 2 3 5 or 10 ml indicates that there is a consistent inhibition of the spreading of India ink with hyaluronidase and in general an increased effect with increasing doses Inasmuch as a twenty four hour period had elapsed after the injection of the adrenal extract it was thought probable that a large part of the

hours Twenty four hours after intravenous injection of adrenal extract in these doses the inhibiting effect was minimal

Inasmuch as the foregoing observations had indicated some specific relationship between hyaluronidase inhibition and the adrenal cortical steroids in both mice and rabbits, we ran a series of experiments to study several pure steroids in an attempt to define more accurately the structural or hormonal factors that were related to this inhibition The primary object of this study was to determine which steroids would cause an inhibition of the spreading reaction when conditions were provided that permitted adrenal cortical extracts to exert maximal effects It was hoped that a better understanding of the adrenal hyaluronidase relationship would be obtained in this way although it was recognized that the conditions of the experiments should be altered in order to obtain complete information regarding the influence of steroids on the spreading reaction As the experiments were designed to obtain comparable data with the different steroids it was necessary to test all compounds under identical conditions For example there was some indication that the adrenal cortical extracts exerted their maximal influence on he spreading reaction within a period of from one to six hours following administration of the extract Since it was the primary objective of this investigation to study compounds that might exhibit the adrenal cortical effect the steroids were all administered within this time interval prior to the injection of the hyaluronidase India ink mixtures The general plan of the experiments was to inject the steroid into normal or adrenalectomized mice After an interval of one to six hours bilateral intradermal injections of India ink and hyaluronidase or saline were made One hour later the mice were killed and the area of spreading was measured The volume of the steroid suspension injected was either 0 5 or 1 ml and the amounts of steroid injected varied from 0 5 mg to 5 mg in the various experiments The steroids were dissolved in sesame oil dissolved in absolute ethanol and then diluted with water to make a suspension in 10 per cent alcohol or a suspension was made by thorough grinding and diluting with 0 9 per cent sodium chloride The alcohol water suspensions were very poor but the plain saline suspensions appeared well dispersed although the steroids settled out rapidly since no suspending agent was used

Figure 28 shows the inhibition of the areas of hyaluronidase enhanced spreading by compounds E A and F in normal mice While the quantities of steroid available at that time limited the studies so that it was impossible to establish accurately the relative activity of these steroids it can be seen that A E and F are all active in inhibiting the area of spread and there is some suggestion that they may be



PER CENT INHIBITION = $\frac{\text{CONTROL AREA} - \text{TEST AREA}}{\text{CONTROL AREA}}$

Average Values are given A control was done in conjunction with each test study

FIGURE 27*

appear also to be necessary in the treatment of rheumatoid arthritis by cortisone These studies presented are representative of numerous other investigations in rabbits and while they do not permit an interpretation of the mechanism of action of the adrenal hormones they do confirm the earlier observations on mice

Thorn May I say before you go on that I do not feel it is fair to imply that with intravenously administered extract the evanescent effect is accounted for by the excretion of the hormone in the urine In most studies which we have carried out and from observations of others I judge that one rarely demonstrates more than 10 per cent of intra venously administered hormone excreted in the urine It is probably inactivated and not excreted in an active form Would you agree with that?

Sayers Yes

Thorn It is interesting that it is not excreted in the urine in large quantities We have tried to recover appreciable amounts of extract in the urine of dogs given relatively large quantities of hormone intra venously but have rarely obtained more than 10 per cent

Pincus A low urinary recovery applies to cortisone administration also

Selye Ten per cent is a very high figure

Thorn Generally it is 3 per cent The maximum rate of excretion would still not account for this type of disappearance curve

Opsahl We did not carry these experiments beyond twenty four

* Reprinted from Yale J Biol & Med 21 255 (1949)

sibility of competitive absorption as will be pointed out later in intra dermal experiments where F is more slowly absorbed This particular dosage level was selected because of the results noted with its use in some *in vitro* experiments

Conn These are all the free compounds?

Opsahl With compound E we used both the free compound and the acetate With compounds F and A we used the free compounds only

Thorn Is the acetate any better?

Opsahl Approximately the same From numerous experiments with testosterone estradiol benzoate progesterone and pregnenolone it ap peared justifiable to conclude that under the experimental conditions employed these steroids did not definitely affect the spreading reaction The lack of activity observed for these sex steroids should be inter preted cautiously However the dosages employed for the hormonally active steroids were large enough to give the conventional end organ responses—actually the dosage of estradiol benzoate and of progester one was much larger than is required for maximal hormonal responses The time of action was far too short for these hormonal effects to be manifested fully and it is possible that different results might have been obtained if a longer time interval had been used

Selye Pregnenolone had no effect?

Opsahl Practically none

Selye How much estradiol was given?

Opsahl One to 5 mg per mouse

Selye A milligram of estradiol will cause tremendous stimulation

Opsahl Perhaps it was a time relationship variation In the case of the adrenal steroids the time relationship was compatible with that required for a primary hormonal response

Selye We ran into that same difference in the antiarthritic effect If adrenal enlargement is produced as a result of muscular exercise arth ritis is inhibited

Long Is it not true Dr Selye that after a period of intermittent exercise you have an enlarged adrenal still rich in lipid material whereas after estradiol it is enlarged but almost depleted of lipid? This might account for the differences in response

Selye During the alarm reaction the adrenal becomes depleted of lipid granules but upon continued exposure to stress the lipid granules return into the cortical cells Conversely with estradiol you can main tain the adrenal continuously in a degranulated condition (5) Does not this difference in the quality of the adrenotropic stimulus suggest the possibility of essentially distinct corticotropic stimuli?

Long The effect of the female sex hormones on the adrenals is very

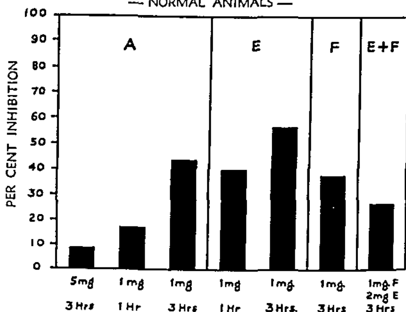


FIGURE 28

quantitatively different in degree of activity Since it was necessary to use aqueous suspensions as vehicles of administration the marked difference in absorption rates that might result from the physical character of the suspension made it impossible to explain the differences on the basis of chemical structure alone However it would appear that smaller quantities of compound E were more effective in inhibiting the spread in shorter time intervals after injection than were compounds F and A *One of the remarkable features of cortisone therapy* is its rapidity of action This characteristic is apparent not only in certain primary hormonal responses such as glycogen storage but also in its inhibition of hyaluronidase enhanced spreading It should be noted that quantities of compound A of less than 1 mg are quite ineffective In an attempt to stimulate the action of lipoadrenal extract a group of animals was given 1 mg of F and 2 mg of E in one dose—the results were surprising in that the degree of inhibition produced was less than that found with either compound alone Our supply of compound F and also of compound A was very limited I wish to point out that the results in this group of animals should not be considered on a quantitative basis Also the toxicity of such a large dose in 25 gm mice may have accounted for the results One cannot rule out the pos

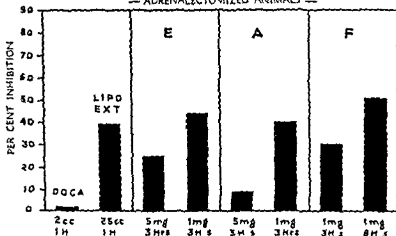


FIGURE 29

control groups were adjusted in such a manner that they comprised an equal number of males and females

White Dr Duran Reynals in his review points out that in general the male animal shows a lesser degree of the spreading effect

Opsahl We have not substantiated that

White Duran Reynals work was in rabbits

Opsahl Figure 29 shows again the inhibition of the areas of hyaluronidase enhanced spreading by compounds E A and F in adrenalectomized mice As we pointed out a moment ago the sex steroids were without effect in the normal mouse and similar results were obtained with their use in adrenalectomized animals Under these conditions any inhibitory effect obtained was without mediation through the adrenal The lack of effect of desoxycorticosterone acetate is shown and the marked inhibition produced with 0 25 ml lipoadrenal extract after one hour absorption time should be noted The activity of compound A in the larger dosage is evident and the complete lack of effect in the smaller dosage levels It is interesting that compound F exhibited a much more marked inhibitory effect after a long period of absorption time I do not have too much basis for speaking about absorption rates of F with the limited quantities we have had available and I realize this is in contradiction to Dr Thorn s comments yesterday of its being the more quickly absorbed We should like to continue these studies with compound F when it becomes available

interesting and as far as I know has not been adequately investigated

Ihorn Could you postulate another possibility? If estrogenic material itself were anticortisone like in effect and also caused enlargement of the adrenal, the net change from these two antagonistic effects might be zero and one would assume that there had been no effect on the level of adrenal cortical secretion

Selye I have investigated given estrogens with cortisone in both the adrenalectomized and intact rat

White Dr Dougherty you had some data at Yale on the effects of estrogen?

Dougherty The experiments you refer to involved a study of the chronic effects of estrogen treatment Estrogen administration increases the size of the adrenal enormously but also results in a considerable necrosis of adrenal cortical cells within four to six weeks after start of treatment From the histological picture I suspect that many of the cells of the hypertrophied gland are not functioning Adrenalectomized mice are very sensitive to estrogens and usually die within a short time after treatment During the period of survival however they develop lymphocytosis which might indicate a rapid utilization of adrenal cortical hormones remaining in the tissues after adrenalectomy (6)

Long Miss Fry and I showed some years ago (7) that stilbestrol, for example caused an increase in liver glycogen some hours after the injection of amounts in the order of milligrams This was not observed in hypophysectomized rats

Dougherty It is possible that estrogens themselves impose a requirement for utilization of hormones of the adrenal cortex If so the increased amount of adrenal cortical hormone secreted following estrogen treatment may be proportional to the demand for utilization and thus excess adrenal steroids are not available for other adrenal cortical hormone functions We have evidence that female adrenalectomized mice require more cortisone to maintain lymphatic tissue within normal range than do male animals (8) Also more hormone is required to protect sensitized adrenalectomized females maintained on cortisone against anaphylactic shock than is necessary in similarly treated male mice *

Astwood I would like to add that toxic doses of stilbestrol are not effective in rheumatoid arthritis

Opsahl I have forgotten to mention a point of interest We investigated whether there was any sex difference in relation to the degree of spreading produced under the various experimental conditions Although this was not found to be the case all the experimental and

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* Unpublished data

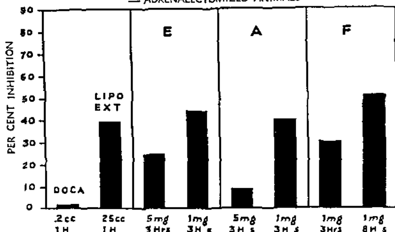


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* Unpublished data

before the injection of the ink mixtures and into the same site This was done in one flank while the opposite flank was injected with an equal amount of saline and the ink mixtures as a control In the representative experiments reported the dose of adrenal extract was 0 025 ml The amounts of hyaluronidase or saline were cut in half so that the volume of the inoculum was either 0 05 ml in the case of the simultaneous injections or 0 025 ml when the injection of the extract preceded that of the ink mixtures These experiments were so designed that the animals were killed following noted time intervals of spreading and all measurements were made from the under surface of the skin The pronounced inhibiting effect produced by the intradermal administration of ACE is well illustrated and the lack of inhibiting effect of DCA and Merthiolate is seen again It is of interest that in experiments where India ink solutions were not subsequently injected it was found that the adrenal cortical extract was absorbed the most

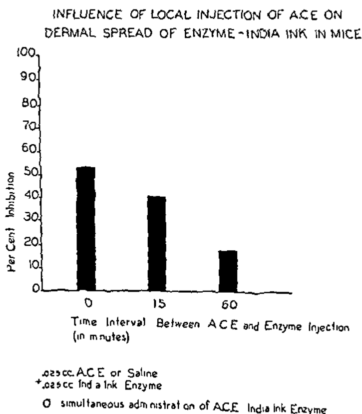


Figure 31

We might just point out that the results obtained were suggestive of a correlation between the C 11 oxygenated adrenal steroids and inhibitory activity in the hyaluronidase enhanced spreading phenomenon Of the numerous steroids studied only three E, F and A have definitely shown activity Furthermore these compounds are the only ones in this series that have the carbohydrate metabolism characteristics of the C 11 oxygenated adrenal steroids

We were curious as to whether or not the inhibiting effect of adrenal cortical extract would be exhibited after local as well as after systemic injection and wished to determine the possible mechanisms of action Several different groups of experiments were carried out in which varying amounts of ACE or saline were injected intradermally into mice at the same site of the injections of India ink with or without hyaluronidase and the effects of variations were tested Figure 30 shows the changes in the dermal spread of India ink as influenced by the local injection of ACE or saline together with hyaluronidase in normal mice In these experiments the ACE was injected intradermally simultaneously or as will be seen in the following figure some time

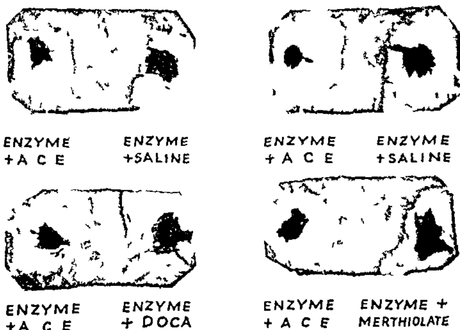
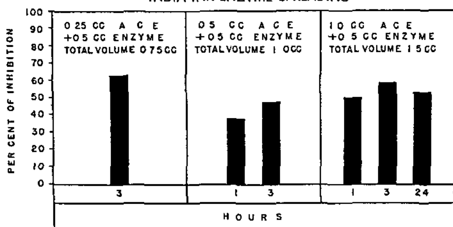


FIGURE 30 * Photograph showing changes in the dermal spread of India ink as influenced by the local injection of ACE (or saline) together with hyaluronidase in normal mice The lack of an inhibiting effect of DCA or methiolate is also apparent

* Reprinted from *Y J B I & M d* 21 48 (1949)



PER CENT INHIBITION = $\frac{\text{CONTROL AREA} - \text{TEST AREA}}{\text{CONTROL AREA}}$

Average Values are given A control was done in conjunction with each test study

FIGURE 32*

occurs If only a 50 per cent inhibition of enzyme occurred that would mean 50 per cent of the enzyme was left to produce an effect That is not what happened for the areas of spreading remained relatively constant and this 50 per cent inhibition of spreading may more properly be interpreted as an almost complete inhibition of enzyme The percentage inhibition presented in all these studies is a relative figure and as there is always a certain degree of spreading when saline and India ink intradermal injections alone are employed it is apparent that 100 per cent inhibition would be impossible to obtain However on a theoretical basis it is likely that these substances have brought about approximately complete inhibition

We have shown that the C 11 oxygenated steroids systemically are the effective components of the adrenal extracts in causing the inhibition of hyaluronidase enhanced spread A more objective means of comparing the relative hyaluronidase inhibition characteristics of these adrenal and related sex steroids can be gained by the technique of local intradermal injection In this technique one deals more directly with the influence of the steroid on the enzyme and the results will not be influenced as much by factors such as crystal size solubility and mobility of the steroid in the tissues This technique of course will be of little value if one is dealing with an effect of an inhibiting agent that is

rapidly and Merthiolate the least rapidly from the intradermal site of the injection

In Figure 31 the percentage inhibitions are shown of results obtained with local administration of ACE at varying time intervals prior to the injection of enzyme India ink mixtures The greatest inhibiting effect of ACE is obtained when it is injected simultaneously with the enzyme and as the time interval between the ACE and the enzyme was increased the inhibiting effect was decreased

Pincus Is this the result of parenteral administration?

Opsahl No intradermal This would indicate that ACE is acting on the hyaluronidase system One might postulate that the adrenal extract does not bring about its inhibition by an alteration of the substrate but rather by an alteration of enzyme activity for one would expect that an alteration of the characteristic of the cement substance would persist for a short postinjection interval such as sixty minutes Had that been the mechanism of action one would expect a marked inhibition to have resulted On the other hand if the ACE were acting directly on the enzyme you would expect the results obtained and as it is obvious there is a rapid dissipation of ACE from the site of injection after sixty minutes the concentration of ACE would be decreased considerably from that originally present

In Figure 32 are shown the results of some experiments with rabbits where ACE was administered intradermally The total volume of control and test solutions in these experiments was always similar and the amount of enzyme in all cases was 0 5 ml The amounts of saline or ACE added vary in the cited experiments When the effects of the 0 25 ml doses of ACE were investigated the extract was mixed with the saline or enzyme solutions with India ink prior to injection With the other dosages employed the ACE was injected immediately before the injections of enzyme or saline and into the same intradermal site The premixed ACE enzyme solution caused a degree of inhibition of hyaluronidase that was equal to or greater than that obtained when larger doses of ACE were administered separately Another point of interest is that the effectiveness of locally administered ACE did not diminish with the time elapsing after injection This is contrary to the observation with intravenously administered ACE

In these studies we are actually measuring the inhibition of spreading areas but many interpretations I make will be presented as though steroid were inhibiting the enzyme hyaluronidase This is not a mere assumption and further evidence in this respect will be brought out shortly In the groups of experiments employing a total volume of 1 5 ml and time intervals of spreading of one three and twenty four hours a blocked spread of approximately a 50 per cent inhibition

ectomy is so effective in increasing the spreading decreased adrenal hormone production could explain that effect

Opsahl We did not pretreat the animals with steroids for several days

Thorn With repeated injections and with intact adrenals I do not see that one can prove this point Those were one hour studies were they not Dr Selye?

Selye Yes If adrenal stimulation by heat for instance acts as do the 11 oxy compounds then adrenal depression by desoxycorticosterone should have an inverse effect Dr Sayers has shown that the insulin sensitivity of DCA pretreated animals is probably increased by this mechanism We have shown that the hyperglycemia produced during the acute phase of alarm reaction caused by any means is inhibited by DCA

Ingle Do you find that testosterone makes the normal animal behave as though it has adrenal cortical insufficiency as you and Dr Sayers have shown to be true of DCA?

Selye We have not tried that I may say that testosterone inhibits the adrenal hypertrophy occurring during the alarm reaction much more effectively than does DCA at equal dose levels If one is interested in blocking the ACTH discharge mechanism testosterone is worth studying

Dougherty Would it not be rather difficult to demonstrate an inhibition below a certain minimum amount of spread? This might be a factor of limitation of the method

Opsahl Yes That brings up the point again that these percentage inhibitions of spreading actually represent closer to 100 per cent inhibition of enzyme because as seen in the controls alone there is always a certain degree of spreading A further clue was afforded by results of studies showing that the steroids and extracts when given locally were much more potent than when given at a distance This again indicates the possibility of their having a direct inhibition on the enzyme system

While it had not been possible to postulate the mechanism of action in the adrenal hyaluronidase relationship it was conceivable that the inhibition phenomenon represented the action of hormone upon the hyaluronic acid through an adrenal liberation of ascorbic acid or through some relationship thereof Another approach to interpretation could be that the inhibition represented an indirect effect of hormone on enzyme through the intermediation of substances liberated by tissues affected by the hormone It was an intriguing possibility that the inhibition of the spreading reaction represented a direct inhibition of hyaluronidase rather than an effect on a specific tissue structure such

mediated through the adrenal and therefore results can be interpreted only on the basis of a direct inhibition of the enzyme The general procedure was similar to that previously described

Figure 33 is a graph showing the percentage inhibitions produced by injection of these steroids The related sex steroids are completely without inhibitory effect Compound A was without effect at this dose level and time interval The marked degree of inhibition noted with compound E should be compared with that seen with compound F at the one and sixteen hour time intervals of spreading From the percentage inhibitions noted with cortisone at both the one and sixteen hour levels this represents an almost complete inhibition of the enzyme

White You have not tried these other steroids in larger doses?

Opsahl Not in the mouse Dr White That was the maximal steroid dosage for the volume intradermally injected A larger volume than 0 05 ml brings about technical difficulties In the rabbits we have employed dosages of 0 5 mg of steroid intradermally in a total volume of 0 5 ml with essentially the same results In the mouse we have given up to 5 mg of these steroids systemically With the related sex steroids there was no effect in any of the various experimental conditions with the time intervals used

White Five milligrams in the case of which steroids?

Opsahl All the sex steroids tested They were without effect

Selye Have you tried pretreating the animals with steroids for several days? The reason I am particularly interested is that both DCA and testosterone cause very appreciable adrenal trophy Since adrenal

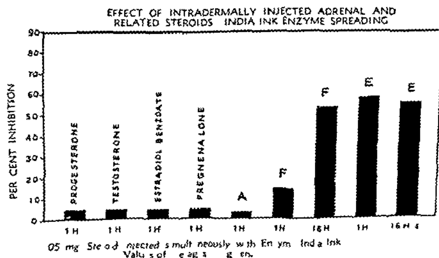


FIGURE 33

activity Calculations of enzyme activity are all made in terms of increase in reducing substances and are expressed as glucose equivalents These studies are not presented as conclusive but I offer them at this time for your criticism It must be pointed out that the development of the method and the standardization of procedures were not only difficult but controls had to be devised for each variable and possible source of error The insolubility of the pure steroids (as we did not have access to the water soluble glycosides) was a definite limiting factor but here again the numerous control series and repetitive experiments served to lessen the possibility of error The use of oily substances in an aqueous system was open to question However the use of chlorobutanol—the solvent for the lipoadrenal extract—as well as controls run with cottonseed sesame and peanut oils decreased the likelihood of misinterpretation of results We studied the effects of enzyme and substrate with respect to source preparation and varying amounts of each the influence of varying time of incubation and temperature buffer strength with and without NaCl and the relation of steroid reducing effects in combination with all of the above—each combination minus one factor of the system The procedure applied in most of the studies presented here involved using 1 mg enzyme 3 mg hyaluronic acid 37° temperature potassium acetate buffer at pH 6 with 0 15 M NaCl and an incubation period of one hour The results of a few of our experiments under some of the conditions tested are summarized briefly

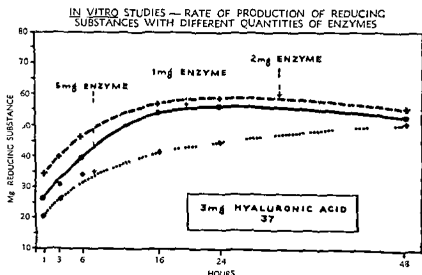


FIGURE 34

as the ground substance

In the studies mentioned previously there was no conclusive evidence that would permit a differentiation of the effect of adrenal fractions on the enzyme or substrate The influence of both adrenalectomy and adrenal cortical extracts or effective steroid fractions on the spreading reaction was observed in both saline and hyaluronidase injected animals From the evidence presented by Meyer regarding the normal presence of hyaluronidase in the skin it is possible that the adrenal effects observed in the saline treated animals were due to an influence on the hyaluronidase system Three observations made it appear likely that this was the case first when adrenal extracts or effective steroids were administered systemically the inhibiting influence of the spreading reaction was considerably more marked on the animals treated with hyaluronidase than with those treated with saline only Second the release of inhibition that was observed in the adrenalectomized animals was considerably greater in the animals treated with hyaluronidase than those receiving saline alone This is particularly evident when spreading is measured a short time following intradermal injection of the dye substances And third a remarkable effect of adrenal cortical extracts or C 11 oxygenated steroids was demonstrated when these materials were injected intradermally and minute dosages caused a rather large decrease in spreading When administered locally the greatest effect is obtained when the ACE or effective steroids are injected simultaneously with the India ink The effectiveness decreases progressively as the intervals between injections are increased This would further indicate a direct action on the enzyme system However as previously stated this evidence could be considered only presumptive and a more complete elucidation of these points was necessary

On several occasions during the course of this discussion I have referred to the inhibition of the enzyme hyaluronidase by certain steroids The evidence for this effect comes in part from *in vitro* studies that we have been conducting during the past two years

The general technique involved in these *in vitro* studies has been the establishment of the rate of hydrolysis of a semipurified hyaluronic acid preparation by a commercial hyaluronidase Hydrolysis of the substrate was followed by the measurement of the increased reducing action due to the end products of hyaluronic acid breakdown employing an alkaline copper reagent the method being patterned after the procedure of Pucher and Vickery Inasmuch as the steroids employed as well as components of the enzyme reaction other than the end products of hyaluronic acid hydrolysis have rather strong reducing properties in themselves detailed check analyses were made so that appropriate corrections could be applied in the calculation of hyaluronidase

evidencing steroid inhibition similar to those obtained at longer time intervals Only three levels of enzyme and one concentration of hyaluronic acid are shown here but we have carried out series of groups of experiments using from 0 01 mg to 10 mg of enzyme—above 3 mg one must use a protein precipitant We have varied the quantity of hyaluronic acid in each test tube from 1 to 16 mg as is shown in Figure 35

Pincus Why not use other measurements of hyaluronidase activity e g viscosity or turbidity changes?

Opsahl Turbidity studies are complicated by the definite insolubility of the steroids In terms of all factors to be considered we thought the reducing sugar method more applicable for our purposes With umbilical cord and vitreous humor hyaluronic acids the results of viscosity *studies vary widely in terms of release of reducing substances there is* but little difference between the two as we proved experimentally This brings up features of another phase of these investigations

Figure 35 shows that the quantity of reducing substances produced are proportional to the hyaluronic acid We have thoroughly tested both umbilical cord and vitreous humor hyaluronic acid in this complex system While this curve represents umbilical cord hyaluronic acid we have established the same relationships with vitreous humor hyaluronic acid The limiting factor in the use of greatly increased amounts of hyaluronic acid in this system is its solubility The well established reducing properties of adrenal cortical steroids made it necessary to

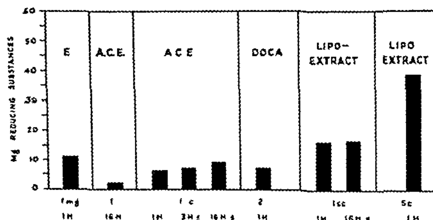


FIGURE 36

In Figure 34 is shown the rate of production of reducing substances with different levels of enzyme over increasing periods of time After six hours the curves begin to level off Furthermore as the amount of enzyme is increased the curves are roughly parallel and there is a comparable increase in amount of hyaluronic acid hydrolysis While it might appear wise to select a time where the rates form a plateau we used a one hour time interval for most of the studies on steroid inhibition shown here One reason for the short time interval was that we wanted to avoid the possibility of bacterial growth Toluene could not be used because steroids would partition into it At the one hour interval the degree of hyaluronic acid hydrolysis in the presence of the three levels of enzyme are *different and parallel effects are* found at the longer time intervals This observation is of importance since if these three curves all came together at one hour so that there was no difference in the liberation of reducing substances it would be impossible to detect quantitative differences in steroid inhibition at this time interval Inasmuch as the three curves are separated and roughly parallel this time interval proved a satisfactory choice and gave results

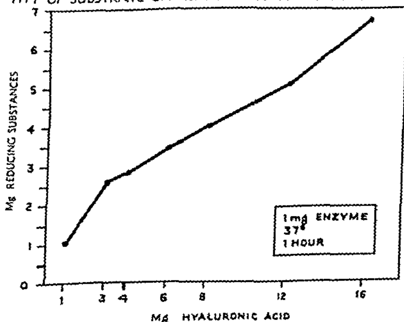


FIGURE 35

ties to be troublesome Appropriate control blanks were run at the same time as the experimental studies

Figure 37 is an illustration of experiments showing the inhibition of hyaluronic acid hydrolysis by hyaluronidase in the presence of lipo adrenal extract at three levels of enzyme Increasing the amount of enzyme from 0 1 mg to 1 0 mg, and maintaining the same quantity of hyaluronic acid produces a nearly threefold increase in amount of reducing substances released At the lowest enzyme concentration there has been essentially a complete inhibition but as the amount of enzyme is increased one is providing an excess of enzyme over that which can be inhibited and as the amount of enzyme was increased the inhibition was overcome This is further evidence favoring the concept that adrenal steroids may be directly inhibiting the enzyme

Figure 38 shows the relative activities of adrenal extract in terms of

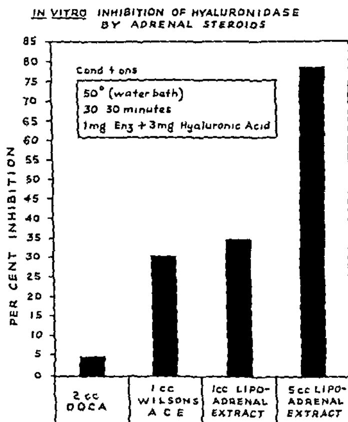


FIGURE 38

establish appropriate blanks and Figure 36 shows the amount of reducing substances produced in the system we used by the various steroids or adrenal preparations We have increased the complexity of the system by adding the components one at a time to the steroids and found that the magnitude of the blanks did not change appreciably, save that 1 mg hyaluronidase added to steroid caused a slight increase in the reducing properties of the steroid It must be emphasized that these particular studies are only indicative Lipoadrenal extract, particularly in the 0 5 ml quantity showed large enough reducing proper

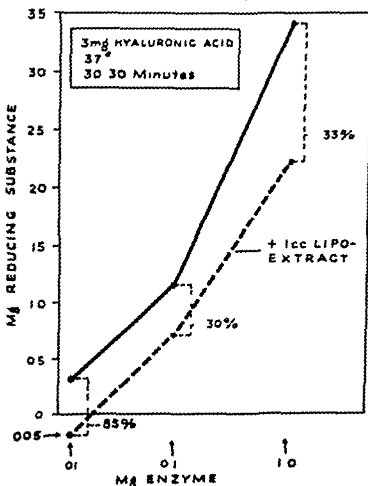


FIGURE 37

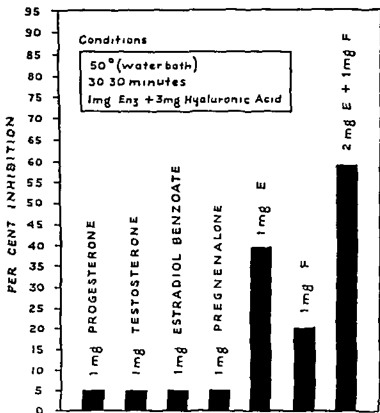


FIGURE 39

further strengthen the possibility that the action of the adrenal steroids on hyaluronidase is at least in part one of direct inhibition

It is apparent from these studies that the adrenal steroid hyaluron idase relationship is a normally functioning physiological mechanism Furthermore it is possible that some of the pharmacologic effects that are observed as a result of the administration of extremely large quan tities of these hormones can be related in part to their influence on this complex enzyme effects system Much more information will have to be collected before it can be established with any certainty how the hyaluronidase system functions in maintaining *the integrity of the* mesenchymal components of tissues and their relations to membrane and tissue permeability and other physiological processes

percentage inhibition of reducing substances released using 1 mg enzyme and 3 mg hyaluronic acid In this system 1 ml Wilson s extract or 0 1 ml lipoadrenal extract produce routinely a 30 to 35 per cent inhibition of release of reducing substances Control studies employing hormone alone and with each variable separately were made and corrections applied in all cases There was a marked degree of inhibition when 0 5 ml lipoadrenal extract was used as is shown in the figure The complete ineffectiveness of DCA is also evident This should perhaps be considered in relation to the data given in the next figure in which a number of pure steroids have been compared as to their activity in these respects

White Do you mix enzyme extract and substrate and put them in the bath or do you preincubate enzyme with extract and then add substrate?

Opsahl We have done it in all these ways However standardizing the procedure we mix enzyme and buffer in the bath and add hyaluronic acid after the first period of incubation In the test systems we add the adrenal extract or steroid plus the enzyme plus buffer and add the hyaluronic acid at the stated time interval Control experiments are of course always done at the same time In addition we have tested the reducing effects of the steroids or extracts separately with buffer solution with hyaluronic acid and with the varying amounts of enzyme used

White If you mix the extract or steroid with enzyme and incubate is there any change in reducing capacity of the steroid?

Opsahl At this time interval of incubation there is with 1 mg of enzyme a very slight increase With lesser amounts of enzyme there is no change from the results found with reducing steroid alone

Figure 39 gives a diagrammatic representation of experiments showing the marked *in vitro* inhibitory effect of hyaluronidase activity by compounds E and F There is some indication that F may be a less active inhibitor under these conditions than is compound E however further studies should be done The related sex steroids were completely ineffective in these studies Relatively little emphasis can be given to the results of the combination of E and F except that it may simulate to a rough degree the amount present in 0 5 ml of lipoadrenal extract These results appear comparable to those obtained with that amount of lipoadrenal extract accounting perhaps for the major inhibitory factor of that substance

It must be emphasized that these *in vitro* studies are presented only as further evidence of the possibility that the action of the adrenal steroids as studied may be directly on the enzyme hyaluronidase Although they should not be regarded as conclusive they serve to

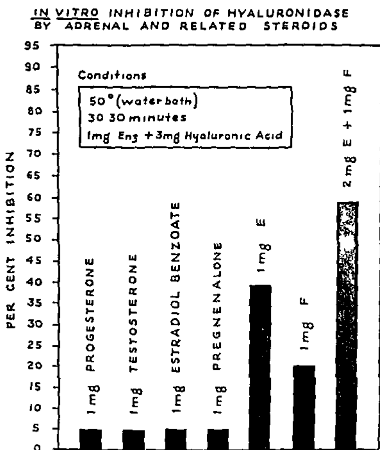


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DISCUSSION

Bloch Have you tested compound A?

Opsahl Not in *in vitro* studies

Thorn I should like to refer to Figure 40 and make a calculation which is of some interest in view of Dr Opsahl s observations on the mouse It is evident with the control and the hyaluronidase material that 40 mg of ACTH per day causes considerable inhibition of the spreading effect of hyaluronidase Cortisone in a dose of 200 mg per day also caused an appreciable inhibition of hyaluronidase spreading action Three days after discontinuing cortisone the hyaluronidase activity had returned to normal In the mouse Dr Opsahl showed us that 0 05 mg of compound E in a 25 gm mouse gave a definite effect locally With 200 mg of cortisone the concentration works out in a 100 kg man to be approximately the same order of magnitude of hormone as you used in the mouse The comparative effectiveness of cortisone in these two entirely different species is of some interest

Loeb What dye do you use?

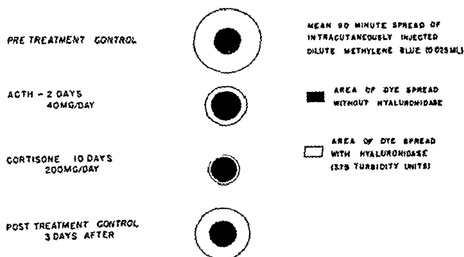


FIGURE 40

Thorn We use methylene blue

Selye Mixed with the hyaluronidase?

Thorn Yes Figure 40 shows the control using only the dye and the dye plus hyaluronidase When ACTH or cortisone are given for ten days in the same individual no spread of the dye occurs

Ralli These were injected systemically not locally?

Thorn Yes

Selye There is considerable literature on the so called intradermal salt test The test is used clinically for diagnostic purposes and might serve as an index of corticoid activity in man What would you think of it as compared to the eosinophil response?

Thorn Our difficulty with all of the intradermal tests is the problem of standards I believe these tests are of value only when carried out on the same individual under the same clinical circumstances

Ralli If an animal were rendered allergic to some substance would this in any way affect the area of spread?

Opsahl I believe you would find an increased degree of spreading activity

White The question arises as to how valid the local skin testing is in an emotionally excitable person?

Loeb We have found no inhibition of the spreading reaction in patients with rheumatoid arthritis treated with doses of ACTH or cortisone sufficient to cause symptomatic remission Salicylate on the other hand in dosage leading to much less clinical improvement caused marked inhibition of the spread with Wyeth s hyaluronidase

Thorn Dr Dietrich Stahelin carried out some studies using a very carefully controlled technique Although these studies may not have any great quantitative significance they did indicate the influence of adrenal cortical hormones on the spreading induced by hyaluronidase

Dougherty It should be emphasized in regard to the use of both the eosinophil and lymphocyte response that the patient should serve as his own control

Thorn The ideal subject again in all of these studies is the patient with Addison s disease where one does not have to consider changes in endogenous adrenal hormone production

Loeb What kind of hyaluronidase did you use and how much Dr Thorn?

Thorn We used 3 75 turbidity units of Wyeth s Hydase

White There is a point which I think is very important in the use of methylene blue It is actively altered by tissues Perhaps that explains some of the differences Did your group Dr Loeb use methylene blue?

Loeb They used T 1824 that is Evans blue

Loewi Methylene blue is reduced by different tissues to a different

degree partly dependent on their different metabolic states We have to take this fact into account if we use methylene blue

Long That is interesting because we have of course discussed the possible application of this to humans and considered these dyes I think in our own mind we ruled out methylene blue for the reasons that Dr Loewi says Obviously you cannot use India ink in man unless you are going into the tattooing business!

Thorn I believe the methylene blue technique which we have employed would be worth testing in other human studies

Selye Hemoglobin is another possibility Stimulated by Dr Opsahl s observation Dr Ducommun in our laboratory has done experiments in rats which confirms Dr Opsahl s work The difference in technique was that he injected subcutaneously rather than intracutaneously Everything Dr Opsahl reported was confirmed except that with DCA LAP and somatotropin he obtained an increase in spreading

Long I would like to obtain some information on the relation of hyaluronidase to connective tissue Do you regard hyaluronidase as an active enzyme of certain types of connective tissue cells which under appropriate conditions is liberated and then attacks the cement substance thus increasing the permeability? I have no clear concept as to the source of hyaluronidase under what physiological circumstances it is released nor where its normal point of action leading to increased permeability is located

Bauer I know of no one who has isolated hyaluronidase from either synovial tissue or synovial fluid although it has been looked for in both normal and diseased states nor have we succeeded in isolating it from either the synovial tissue or the synovial fluid of rheumatoid joints The synovial fluid in some of these cases showed a marked reduction in hyaluronic acid and also showed very low viscosity yet hyaluronidase activity could not be demonstrated The latter findings whether the result of increased or decreased enzyme activity can be corrected by the administration of either ACTH or cortisone If hyaluronidase is present in rheumatoid joints it is most elusive The same is true of normal joints Our experience with hyaluronidase skin tests has been similar to that reported by Dr Loeb We too dropped it because of the inconsistency of the results

Long Do I understand that hyaluronidase has not been isolated from connective tissue?

Bauer Not from synovial tissue or synovial fluid

Opsahl Meyer has reported good evidence for the presence of hyaluronic acid and hyaluronidase in skin and I think his results are conclusive

Long This is obviously a very important point for Dr Opsahl Is she carrying out an experiment with enzyme isolated from another source using the rabbit skin as a sort of test tube or has this substance any importance in connective tissue physiology? I think the reason she says there is a certain inconclusiveness about this work is that we are not dealing with a pure enzyme We are also not dealing with a pure substrate

Dougherty I wonder if anybody has any information on the chemical nature of the cement substance of the endothelium?

Pincus In regard to your point we have been conducting experiments on the effects of various adrenal steroids on the viscosity reducing action of hyaluronidase and we get no effect *in vitro* even with very large amounts of steroid

The hyaluronic acid came from umbilical cords From this we have prepared in the laboratory certain antihyaluronidases by the chemical treatment of the hyaluronic acid itself There are very potent inhibitors in very small amounts (9) These data seem to me another instance where there is an apparent contradiction The only explanation that I can offer depends as Dr Long points out on the fact that what is usually called hyaluronidase is unquestionably a mixture of enzymes This has been demonstrated by a number of workers and it may be that the viscosity reducing enzyme is different from that which is concerned with the production of reducing substance I would like to ask Dr Opsahl therefore what is the viscosity of the hyaluronic acid which she used? There are some preparations which have no viscosity that is they have just about the viscosity of water There are others which have very high viscosity We have been using in our work for obvious reasons material which has extremely high viscosity and that may perhaps be suggestive in this connection Have you measured the viscosity?

Opsahl Yes in certain of these experiments For the most part the hyaluronic acid preparations have been of low viscosity We have tried to eliminate the possibility of error in these experiments and have tested a number of hyaluronic acid preparations Dr Hadidian has very kindly given us several of his different preparations Some years ago Wyeth gave us preparations both from umbilical cord and vitreous humor sources In passing I might mention that we have ourselves isolated hyaluronic acid both from human umbilical cords and from vitreous humor of cattle eyes As I stated before using the same preparation of hyaluronidase umbilical cord hyaluronic acid and vitreous humor hyaluronic acid give essentially the same results in terms of reducing substances released It has been pointed out by Meyer quite conclusively that this is not the case in terms of viscosity measurements

Bauer What hyaluronidase preparation are you using?

Opsahl We have used a number of standardized preparations supplied us by Dr Seifter of Wyeth Inc They have ranged from impure to highly purified samples and we have tested preparations of widely varying potency

White A classical example as an analogy is in the field of nucleic acids where the depolymerizing enzyme or enzymes are quite different from the hydrolytic systems

Pincus Evidence would indicate that in the progression of hyaluronic acid down to the sugar units the first effect is due to a depolymerase a separate enzyme There may be one two or more other enzymes concerned with the successive cleaving of the sugar molecule (10) Actually the cleavage ultimately does go down—how we do not know of course—to the disaccharide unit or monosaccharide but as I understand it the depolymerization ordinarily precedes the cleavage of reducing substances

Long The experience of others has been that as you progressively attempt to purify these substances you have largely a depolymerase

Pincus We think it is the depolymerase that has the marked spreading action in extremely low concentration

Loewi We ought to look for other substances which have an inhibitory influence on the spreading factor I do not think it was only this one Do you know the literature about this Dr Opsahl?

Opsahl I believe so At least I have tried to keep up with it

Loewi Did they not try other substances which would inhibit the spreading factor?

Opsahl Yes there are a number of them Salicylates are believed to inhibit the spreading of hyaluronidase although there is still some controversy regarding this Heparin is known to be a specific inhibitor of hyaluronidase A possibility of one mechanism of action was that administration or release of adrenal steroids might cause destruction or mast cells and release of heparin which would in turn inhibit hyaluronidase This might in some way fit in with Dr Dougherty s concepts Beiler and Martin have published very interesting papers on the inhibitory action of vitamin P compounds on hyaluronidase They point out that of the vitamin P compounds tested only rutin showed an inhibitory action on hyaluronidase and this only at high concentrations of 1 mg per ml The only compounds possessing activity at the lower concentrations were ascorbic acid and dicumarol However a combination of ascorbic acid and the vitamin P compounds showed a marked potentiation of inhibitory action on hyaluronidase The fact that dicumarol as well as heparin functions as an anticoagulant and that both manifest this property of inhibiting hyaluronidase caused them to

speculate whether the anticoagulating properties of these compounds are related to their effects on hyaluronidase In a later paper they investigated the effect of vitamin P substancs on histidine decarboxylase Mayer and Kull have shown that hyaluronidase is inhibited *in vivo* by antihistaminics and there is now increasing evidence pointing to some relationship between hyaluronidase and histamine We could discuss numerous other investigations but I think we had better stop

Pincus Does vitamin C have a spreading effect?

Opsahl No Ascorbic acid inhibits the spreading reaction

Pincus The effect of ascorbic acid has been reported by Duran Reynals (11)

Astwood Yesterday Dr Bloch pointed out one or two possible exceptions but in general hormones have not been shown to act *in vitro* I wonder whether Dr Opsahl interprets her data as indicating that this is a hormone action on an enzyme or whether it is due to some other property of the sterol such as its polar or lipophilic character or its sugar like side chain—something quite apart from its action as a hormone When you come right down to it 1 mg of inhibitor was used versus 1 mg of enzyme It does not sound like a specific enzyme inhibition by a hormone

Long You have to take the relative solid phase against the phase that is in solution and I think as Dr Kendall remarked here last year that most of these steroids have the solubility of the cement on the pavement It is extremely difficult to get more than small amounts of them into solution particularly in small volumes

Pincus We can show you how to get them into solution We dissolve up to a gram per liter of blood

Long I would agree that it is possible in blood but not in a small test volume such as we used here

Pincus In our experiments we did get a lot into solution

White Dr Dougherty made a comment about the loose use of the word mesenchymal and I wonder if he would indicate what he had in mind for those of us who use it loosely

Dougherty I can only give a very opinionated reply to your question The reason the term mesenchymal disease is inappropriate is that it implies that embryonic cells must necessarily persist and take part in the development of pathological alterations in the adult organism For example we do not call a disease of nervous tissue or skin an ectodermal disease The essence of the matter is found in the fact that cells of connective tissue are capable of performing so many different functions and undergoing so many morphological transformations that we presume that differentiation in the embryological sense must continue to occur in the adult

Bauer What hyaluronidase preparation are you using?

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White A classical example as an analogy is in the field of nucleic acids where the depolymerizing enzyme or enzymes are quite different from the hydrolytic systems

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the other hand such morphological characteristics are lost there would be no way of distinguishing which cell was a mast cell and which a fat cell In addition such connective tissue and reticuloendothelial cells can begin the synthesis of hemoglobin and in this case we say they are erythropoietic Thus the morphologist has developed his theories on the basis of structure rather than function of these cells According to the theory presented above the reactions of connective tissue which are characteristic of physiological as well as pathological processes are not governed by embryonic organizers but rather are a stimulus response type of phenomenon

Loeb I would like to agree completely My point is that chemically there is however a common theme song in all of these derived cells

Dougherty The stimuli are unknown which urge these cells to assume different structural and functional characteristics in various processes we call pathological conditions If we have cartilage forming in our aorta we do no like it If we have an increase in the amount of fibrosis in our livers it is not good If we have adequate wound healing on the backs of our hands and ossification in our growing bones that is one thing If these same processes occur in our coronary arteries that is too bad but essentially the basic process is the same

Long Do you think they can be pushed hard enough to make steroid hormones?

Dougherty I think all steroid hormones are made by modified fibroblasts The fibroblasts forming the capsule of the adrenal the cells surrounding the capillaries in the theca of the ovary the interstitial cells of the testis are all morphologically similar There is a considerable amount of evidence indicating that they all can catalyze fiber formation and be precursors of other cell types (ectopic myelopoiesis etc)

Bauer I wish you had more factual data to back some of your statements When I say factual data I mean data chemical data pertaining to the actual function of the cell because after all you are examining tissue under a microscope and are making certain deductions which you cannot necessarily explain without making certain assumptions What they actually mean chemically speaking is what Dr Loeb was getting at It is the thing we do not know

Dougherty We know a great deal about the functional significance of cellular changes as they relate to over all processes such as inflammation hemopoiesis bone growth etc We know almost nothing about the chemical or physical factors which influence the direction for example in which cells transform and take up various tasks

Ralli You mentioned Paul Weiss work in this connection I believe

Dougherty There is an excellent account of his concept of differen

Loeb Could you not turn that thought around? My basis for talking as an anatomist or embryologist is absolutely zero Yet I wonder, if on the other hand it is not helpful to think of these primitive cells as responding in a specific manner to a variety of chemical stimuli and that in response they come to represent what we consider to be certain differentiated cell types e g fibroblast osteoblasts etc

Dougherty I think Dr Loeb that we are saying similar things but perhaps it would be well to clarify our points of view It would be convenient to agree on a term which would encompass the various reactions of connective tissue which result in both structural and functional pathological alterations However I do not believe that mesenchymal disease is a desirable term Fundamentally the issue to be clarified is the question of what role the various cells of connective tissue play in disease The answer to this question can be sought when we classify the reactions of connective tissue with regard to specific stimuli For example among numerous possible responses to varied stimuli there are two which have engaged most of our attention The first is the appearance of nonautochthonous cells and the second is the participation of native connective tissue cells in disease Study of the first of these responses suggested the necessity for assuming that embryonic or at least undifferentiated cells were available to account for the modified cell population in diseased connective tissue Thus depending upon the cell types appearing in different diseases various theories for the derivation of these cells from lymphocytes and local connective tissue elements have been evolved A prevailing point of view is that such cells are derived from undifferentiated mesenchyme particularly those cells said by Maximow to be located just outside the capillaries

Bauer Are you willing to accept Maximow s concept?

Dougherty No I do not completely accept his point of view The presence of such primitive mesenchymal cells along capillaries is based in my opinion on insufficient evidence My own point of view is that it is not necessary to assume an embryonic type of differentiation for the cells of connective tissue Evidence for such a type of differentiation is purely morphological i e the assumption is made that because one sees morphologically distinguishable cells that these cells represent rigid lines of embryological differentiation Cells of the connective tissue including the reticuloendothelial system have a wide variety of functional capacities and the morphological appearance of any one cell of this system at any frozen moment in history (histological preparation) depends upon the physiological role of that cell at that particular time For example if such a cell accumulates neutral fat it is called a fat cell if metachromatic granules accumulate a mast cell etc If on

fibroblast can turn into any cell

Dougherty Not any cell but many of the other cell types in connective tissues

Selye You do admit it can turn into adrenal cortical cells?

Dougherty I think so

Selye If you admit that fibroblasts can turn into adrenal cortical cells I would like to have a definition of epithelium You must define it on an embryological rather than a histological basis

Dougherty That bothers me every year when I start teaching histology Epithelium simply means a layer of cells having little intercellular cement lining a tube a cavity or a free surface The term is descriptive and does not imply anything related to a special embryological origin

Thorn Do you care to comment on chemotaxis in the differentiation of cell type? Does it come into play at any given stage in any of these cells?

Dougherty The fibroblast displays chemotaxis in one sense that is that it apparently follows fibrin strands and migrates into inflamed areas at the stage of repair The leucocytes are attracted into an inflamed area It is interesting in this regard that PMN s are attracted before lymphocytes even in those species in which 85 per cent of the leucocytes are lymphocytes This certainly tends to show a differential chemotaxis as far as blood cells are concerned I know of no evidence indicating that transformations of one cell type to another are governed by chemotactic factors

Ralli May I ask a question which may be a little beside the point? The capacity of the liver cells to regenerate following removal of part of the liver seems to be influenced by certain nutritional factors Do you feel that that is a direct stimulus to the cell?

Dougherty I just have no knowledge on that The same problem for instance exists in repair of bone The stimuli for repair or regeneration are largely unknown

Bauer In the case of tissue culture the poorer the environmental conditions the more differentiation you get It is most mysterious why under these conditions the formation of cartilage and bone takes place although it is in an environment which leads to death

Dougherty Is not the same thing true in many respects *in vivo* if you diminish the blood supply? Maximow showed years ago that you can convert the kidney to bone simply by severely diminishing the blood supply

Ralli Do certain tissues react differently when you cut off the blood supply? Would the liver tend to develop more fibroblasts? Is there inherent in any type of cell a tendency to do a certain thing in response

tration based on functional and morphological changes in cell types in a paper which is a portion of a symposium (12)

Loeb Dr Dougherty, may I again say that one would probably be willing to distinguish epithelium from prefibroblasts in its capacity to respond to different chemical stimuli, is that not right?

Dougherty Yes

Loeb I think we are getting down to a question of semantics Are we to call what we have primordial epithelium or primordial connective tissues?

Dougherty That gets into the problem of what is meant by differentiation The real question is what the basic cell types of connective tissues do not only in the chemical sense but in more grossly defined ways such as the formation of fibers phagocytosis elaboration of heparin etc

Loeb But the chemical circumstances determine what they do—what they become

Dougherty Yes but we do not know what these circumstances are When we do we will know a great deal about aging and degenerative diseases in general

Ralli Would you define as circumstances the intracellular constituents?

Dougherty Both extra and intracellular constituents—the intracellular constituents are modified by the extracellular However I do not think we know enough about the factors influencing connective tissue cells to go beyond the merest statement of the problem

The problem for instance of the presence of antibodies was brought up by Dr Thorn concerning Dr Favour s work The ubiquitous distribution of lymphocytes which may form antibody makes it possible that antigen antibody union could occur in innumerable sites with ensuing allergic inflammation It is possible that lymphocytes may form antibody I think too much is made of the various theories concerned with the particular cell type which synthesizes antibody Probably all of the connective tissue cells are involved in one way or another The fact that lymphocytes may transform to macrophages and fibroblasts and other cells suggests that it is improbable that one cell type is solely responsible for antibody formation In other words there is a basic cell type with several different functional capacities One of these functions is antibody formation

Loeb You include plasma cells in them?

Dougherty Yes and I think all these cell types may possibly contain antibody Thus the possibilities for antigen antibody union among these cells are numerous

Selye I would like to clarify As I understand you you mean a

Loewi I would like to know whether or not most of you assume that the cortical hormones do act exclusively on mesenchymal tissues

Long Yes Dr Dougherty prefers the term connective tissues

Loewi In my opinion there must be other cells in addition to the mesenchymal cells which are acted upon by cortical hormones Or do you think that the enormous increase of protein catabolism originates exclusively from mesenchymal tissue? What about the striated muscles?

Dougherty Von Mollendorf proposed some time ago that if one eliminated connective tissue and closely related cells one would have skin nervous tissue bronchial tree and a few other structures with nothing to hold them together or supply them with blood

Thorn Most of the effects of hormones are on the skin and nervous system Would you like to comment? I am confused about the question of the relation of the adrenal cortex to pigmentation in man since we see hyperpigmentation in adrenal insufficiency and since hyperpigmentation develops so rapidly with cessation of adrenal hormone administration

Sayers As a point of clarification may I ask this question Does pigmentation take place with an excess of cortisone as well as ACTH?

Thorn Right

Loeb Correlations of pigmentation and cortical function are difficult to make You have all witnessed Addisonian patients who in the course of years even maintained on salt alone have lost their excessive pigmentation including the ink spots in the mouth On the hyperadrenal side we have one Addisonian patient who in the course of about two or three months of treatment with cortisone has lost the ink spots in her mouth which is as rapid a loss as I have ever seen

Sayers May I ask if there are any distinguishing features between the pigmentation of the Addison s disease and that which you induced by the administration of cortisone?

Thorn Most of our cortisone and ACTH periods of treatment are relatively short I have never observed the development of mucous membrane pigmentation with ACTH or cortisone

Loeb No neither have we

White Can we get back to the spreading factor? I would like to ask a question which relates to what Dr Dougherty reported with the detergent and perhaps to the phenomenon of tattooing Is there any effect of the 11 oxygenated steroids on the rate of clearing of a particular area of deposited material e g the removal of ink markings? In other words if you produce granules with the detergent can one accelerate the rate at which that material disappears by subsequent treatment of the animal with adrenal cortical steroids?

Opsahl One can hardly talk about disappearance of the India ink in

to say a decreased amount of circulation?

Dougherty I would put it this way A large part of stimulation to differentiation is a matter of blood supply There is no real proof but plenty of leads which indicate there may be something in this idea The process of aging is toward differentiation of cells and the mani mate i e increased fiber formation An economic example is the dif ference in cost between calves liver and beef liver! You might make a statement to this effect if you have a cell with multipotentialities and the development of each potentiality is dependent upon an adverse stimulus then under the circumstances in which you have maximal ad versity you have maximal bringing out of these potentialities This may have no basis in fact but that is one way of looking at the situation When you have homeostasis there is no need for the cell to do these various things

Bauer Is it not true that many tissues revert to pure fibroblasts if carried long enough in tissue culture?

Dougherty Practically all cells of connective tissue lymphatic tissue and reticuloendothelial cells as well revert to fibroblasts (morpho logically distinguished) There are about fifty years of intensive research which have resulted in a multiplicity of facts and theories Our philosophical job is to forget the old theories and attempt to relate the facts—establish new theories which can be forgotten when they have served their purpose

Loeb I would not like to think that it is all just in terms of oxygen supply I still believe in a so called natural duration of life and I go back to the classical experiment carried out by Jack Northrup and my parent on the life span of Drosophila which was purely a function of temperature You have for each ten degrees increase a 100 per cent decrease in duration of life and for each ten degrees lowering a 100 per cent increase in duration of the life span So I would much rather think in terms of a series of chemical reactions which have to be run in the natural duration of life granted that oxygen supply may modify the reactions to a considerable extent But the over all picture involves integrated chemical processes which are a function of temperature—at least in the Drosophila

Thorn In the clinic one has a good example of an exception to this theory If you start out from birth with a poor blood supply such as is observed in patients with congenital heart disease and cyanosis one does not age more rapidly on the contrary the patient appears more youthful if anything There is no evidence that such patients dry up They die ultimately of heart failure and are still young looking patients

that the variation of results might be explained on this basis and it seems equally possible that their effects could have been mediated through the adrenal The original object of our studies was to determine which steroids would cause an inhibition of the spreading reaction when conditions were provided that permitted adrenal cortical extracts to exert maximal effects—under these conditions in our studies the sex steroids were without inhibitory effect I think that this is further borne out in our studies with adrenalectomized animals and studies involving local administration of the various steroids I cannot explain their lack of inhibitory effect with local administration of compound E We—and I believe others—have substantiated our published results under many different experimental conditions

Dougherty Dr Opsahl have you made any studies on heparin release?

Opsahl Not as yet

Bloch I wanted to ask about your *in vitro* experiments Have you attempted to correlate the action of the various hormones with adsorption on the enzyme?

Opsahl No

Bloch Have you tried to relieve the inhibition by DCA with some other steroid hormones?

Opsahl No

Pincus We have found with the various antihyaluronidases that you can displace them all if you use enough substrate In other words hyaluronic acid will displace deacetylated hyaluronic acid from the viscosity reducing enzyme

Opsahl We have done that We have increased the amount of substrate maintaining the enzyme concentration constant in studying the increased release of reducing substances Also we have maintained the amount of substrate constant and have studied the effect of increased amounts of enzyme from 0 01 mg to 3 mg In the *in vitro* inhibition studies with steroids our data are not complete on this point

Pincus If we are suggesting the combination of what appears to be a sugar like portion of the steroid molecule with the enzyme (which would be the thing one would suspect offhand) can we drive it off the enzyme? With the antihyaluronidases that we worked with we know the exact proportions that are necessary to link up presumably the active centers of the enzyme

Loeb I do not recall in your studies on the splitting of hyaluronic acid what was the quantitative relationship of enzyme to substrate Was it 5 mg of hyaluronic acid and 0 5 to 1 5 enzyme?

Opsahl In the *in vitro* experiments we used 1 mg enzyme and 3 mg hyaluronic acid

the studies however both on systemic and local injection of the C 11 steroids the spreading of the India ink is inhibited When adrenal extracts or effective steroids are introduced locally without India ink the raised bleb disappears almost immediately With control injections of saline or other substances the bleb remains a longer time In relation to Dr Thorn s remark about tattooing Some years ago we considered the possibility of using fluorescin as an effective indicator substance in skin testing in patients Thus far we have tried it only in rabbits but its use might be a solution to the tattooing problem

Dougherty We do not have any information on the role of cortisone in reducing the mycelophagosis

White What I had in mind was that in addition to cortisone inhibiting the spreading of ink would it accelerate removal of previously deposited material?

Dougherty We do not know

Loeb Has there been any further work by Albert on his induction of brown pigmentation in frogs with ACTH? Also have his findings been borne out with purer preparations of ACTH? I think the question raised there was whether the pigmentation which followed ACTH might in reality be associated with some other substance of the pituitary Of course others have raised the question of whether the pigmentation in the Addisonian may be associated with some pituitary factor which is elaborated in increased amounts in response to lack of the adrenal Sprague reported that if I am not mistaken at the first ACTH conference

White Pigmentation develops in tadpoles with ACTH or crude pituitary extract This does not occur with adrenal cortical extract and the effect may be due to possible posterior pituitary contamination

Dougherty It could be the intermediate lobe

Loeb You do not see pigmentation with pure ACTH?

White We get into the question of what is pure ACTH With commercial ACTH pigmentation may occur rapidly in tadpoles

Ingle Dr Opsahl you are probably familiar with the experiments which were carried out at the Merck Institute in which it was shown that a number of steroids other than the 11 oxygenated steroids of the adrenal were able to inhibit the spreading action of hyaluronidase Have you repeated any of these experiments?

Opsahl You are speaking of results with the various steroids?

Ingle Yes Dr Winter did these experiments (13)

Opsahl We had previously published results of some of our experiments and Dr Winter wrote me about their experiments before they were published The time relationships were different in their studies and they gave repeated dosages of the sex steroids—it seems possible

interest Quite naturally we were concerned with these problems in relation to our own studies I believe I am correct in stating Dr Hechter feels that to a certain extent the volume and pressure are instrumental in the degree of spreading produced The two Figures 41 and 42 show some early studies with rabbits We wished to determine whether increasing the volume of injection with ACE or saline as compared to a smaller volume containing the same basic amounts of enzyme or saline would affect the maximal degree of spreading When the volume was increased with saline there was essentially no change in degree of spreading produced As you can see there was a marked inhibiting effect when ACE was administered These preliminary experiments while not in themselves conclusive permit the interpretation that in intradermal investigations the use of increased volumes in themselves are not a limiting factor in accuracy

In other groups of experiments (see Figure 42) 0 25 ml 0 5 ml or 1 ml saline or ACE was injected intradermally followed immediately by intradermal injections of 0 5 ml saline or enzyme solutions with India ink into the same site The total volumes were 0 75 ml 1 ml or 1 5 ml respectively One three or twenty four hours following injection of these test substances the animals were killed skinned and measurements made as described before Not only is the

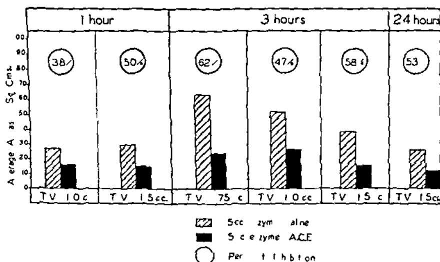


FIGURE 42

Loeb Is that not very high for an enzymatic reaction?

Opsahl The time interval is short and we were interested in obtaining a measurable quantity of reducing substances If less than 3 mg hyaluronic acid is used in this short time interval with either 0 1 or 0 01 mg of enzyme the quantities of reducing substances released are very low It would be interesting to use chondroitin sulfates in *in vitro* tests because of the belief that hyaluronidases may act on them It might aid in further understandings of these problems

White I was going to ask in this connection whether hyaluronidase has any effect on heparin *in vitro*

Pincus Heparin is inhibitory to hyaluronidase In regard to Dr Opsahl s last remark I would think you might have an enzyme in the mixture which attacks the chondroitin sulfuric acid rather than hyaluronidase I would like to ask a question about the *in vivo* work, concerning spreading I am sure Dr Opsahl that you recall some work that Dr Hechter did on the pressure of the bleb in relation to the amount of spreading and that he has consistently said that where you are dealing with an inflammatory reaction you have an effective increase in pressure whereas if you are dealing with noninflammatory reaction you have no effect (14) Is there no accumulation of fluid at the bleb site?

Opsahl We have followed Dr Hechter s work with a great deal of

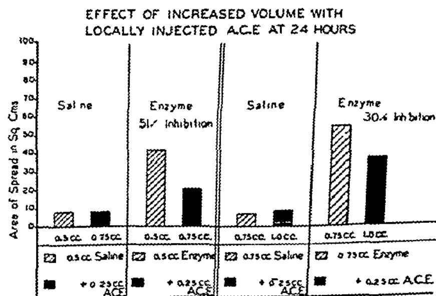


FIGURE 11

ours in regard to testosterone and some of the related steroids

Long There is interest in Dr Seifter s work in relation to yours First of all he states in his paper that these effects are seen either with a freshly removed bladder or after it has been dried to 100° C Of course after drying at 100° C it is just a dead membrane and as such I presume was serving as a source of substrate for the enzyme The hyaluronidase solution was placed outside the bladder which was set up as an ordinary osmometer He states first of all that the C 11 oxysteroids if added to the hyaluronidase outside the membrane reduce the rate of rise in the osmometer DCA on the other hand increases the rate of rise while the blood serum from animals that had been given colchicine reduced the rate of water transfer through the membrane I think he did report some effects with testosterone

Long Here you have what amounts to an *in vitro* effect of steroids on this enzyme

Bauer They are odd experiments You take the same bladder wash it out and dry it and use it for the next hormone

Long Instead of using as Dr Opsahl did a solution of hyaluronic acid he used the hyaluronic acid present in the bladder which was used turned inside out exposing the inside surface to the action of the hyaluronidase

White Quite obviously this technique eliminates the inflammatory reaction since it is a dead bladder

Pincus There you may be dealing with surface action of steroids about which we know too little Certainly steroids affect surface tension in one way or another In other words is he merely making the substrate more accessible on a purely physical basis?

White Were detergents tried to determine whether an effect on permeability occurred?

Pincus My recollection is no

White You may remember in the early phases of our studies Dr Opsahl you made the interesting observation that the same data i e the effect of adrenal cortical extract on spreading could be obtained in the dead mouse You severed the spinal column and did the experiment immediately on the dead mouse When you tried this technique in the adrenalectomized mouse the dead animal was nonresponsive If this still holds it is an interesting example of the relative sensitivity changes which take place in the skin following adrenalectomy Do you still find that you have to do your adrenalectomized mice while they are still alive or can you obtain effects in the recently killed adrenalectomized mouse?

Opsahl That brings up interesting points which I did not feel we had time to discuss before Some four or five years ago when we first

marked inhibiting effect of ACE seen in these experiments but with the increasing total volumes injected there is no clear cut evidence of enhancement of spreading Rather as you see Dr Pincus if one notes the average spreading areas after a three hour time interval with the three different volumes there appears to be a relative decrease in areas of spreading produced with the increasing volumes

Further as may be seen from the results with the 1 5 ml volumes the inhibitory effect did *not diminish with time following injection* and the actual area of spreading varied little In the dosage and volume ranges studied no definite relationship between dosage and inhibition *is to be noted* From the results of these and other similar experiments I do not feel that under these conditions the volume has much to do with the degree of spreading Actually where you are increasing the volume you are decreasing the concentration however a constant amount of enzyme is being introduced in the inoculum Does that answer your question?

Pincus Except that the degree of spread is a function of concentration of the enzyme as well as of the volume Dr Hechter s point is when you get beyond a certain concentration of enzyme then the volume is important The question is in what range of enzyme concentration are you working The reason why I bring up this consideration is obvious If the effect of the adrenal hormones is essentially to reduce the inflammatory reaction then you would get a lesser spread The point behind it is (if Dr Hechter is right) that you have as a factor in spreading the pressure due to the inflammatory reaction If the adrenal hormone interferes with the inflammatory reaction but not with the hyaluronidase spreading will decline

Opsahl We have studied the influence of pressure in certain of our experiments and have found no appreciable differences in degree of spreading with the designated time intervals of spreading Naturally however we have standardized our methods so that difference in pressure is not a variable

Long Dr Opsahl I think before we close some mention ought to be made of the work of Dr Seifter (15) who to my mind has also shown there is an *in vitro* effect of steroids on hyaluronidase at least in some of his experiments where he used a dried bladder of rabbit as an osmometer

Opsahl Dr Seifter took the urinary bladder of the rabbit and used it as an osmometer in an *in vitro* system where he placed it in a large beaker containing fluids to which he added hyaluronidase and varying steroidal substances or extracts He used the changes of osmotic pressure inside the tube connected to the bladder to reflect increases or decreases in permeability I believe that his results differed from

clinical studies or Further clinical studies with ACTH and adrenal cortical hormones ?

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started these investigations we had no standard procedure It was necessary to establish assay procedures that were as free as possible from stress factors that might fire the adrenals and a plan had to be evolved whereby the preparing and testing of the animals could be carried on without assistance and with accuracy First the problem of shaving and injecting the unanesthetized mouse was of concern for if the adrenals were shown to have a relationship to the spreading of hyaluronidase any stress stimulating the adrenals would be a complicating factor In subsequent early experiments we used amytal or nembutal anesthesia However in the dosages used there was sufficient variability among mice so that this amount proved either to be lethal or did not provide sufficient relaxation of the animal to permit testing Inasmuch as those methods offered no satisfactory basic standard for standardization of methods and as it had been reported that the degree of spreading at twenty minutes was the same in killed or living mice or rabbits we used just killed animals for preliminary experiments We recognized however that such test animals offered marked limitations of procedures and interpretations of results When we first used adrenalectomized animals killed just prior to the intradermal injection of India ink and hyaluronidase or saline this method produced bizarre results and it became obvious that the role of the adrenals could not be explained in animals that were dead at the time the spreading reaction was developing It was apparent that some physical or chemical agent that inhibited the spreading reaction was formed in the animals tissues It was impossible to state what this inhibitory factor was but it might well have been one of the products of autolysis some compound resulting from tissue anoxia or more likely the lack of a normal and unimpaired circulation We then turned again to using the living animal and employed light ether anesthesia at the time of the intradermal injections We found that after the first twenty minutes spreading time there were very marked differences in the degree of spreading produced in the living and in the dead animal This makes good sense Moreover we then found a marked enhancement of hyaluronidase spreading in the studies on the adrenalectomized animal We controlled the factor of anesthesia by exposing all animals control and test to the same degree of anesthesia

White I wanted to put this general question or point before the group Why has the adrenalectomized recently killed mouse lost this adrenal cortical permeability relationship whereas it is maintained in the nonadrenalectomized dead mouse? It may be an example of the relatively greater sensitivity of the tissues of adrenalectomized animals to deprivation of hormone

Long Dr Thorn would you proceed to open the discussion on the

alpha rhythm and a decrease in the number of beta waves as well as electrocardiographic changes

I should like to discuss these points individually

CORRECTION OF HYPOGLYCEMIA The capacity of the Addisonian to withstand a twenty four hour fast is markedly enhanced under cortisone treatment (Figure 43) It appears that the improvement represents the summation of several actions of cortisone a) increased glycogen reserves at the start of the fast b) increased rate of glu coneogenesis from protein during the fast c) increased capacity to mobilize fat stores to produce ketones and to utilize fat (Figure 44) Although glucose may be formed in increased quantities from protein under the influence of cortisone our balance studies would suggest that such a metabolic transformation does not provide large quantities of glucose since only a small negative nitrogen balance is observed It is of course possible that some readjustment in protein catabolism and anabolism might have taken place An increase in mobilization and utilization of fat is suggested by the increase in the serum level of ketone bodies and the fall in respiratory quotient observed during the initial period of cortisone treatment

It is obvious that in the absence of the adrenal medulla the capacity of the cortisone treated Addisonian to respond to sudden needs for increased glucose will continue to be impaired because of inability to

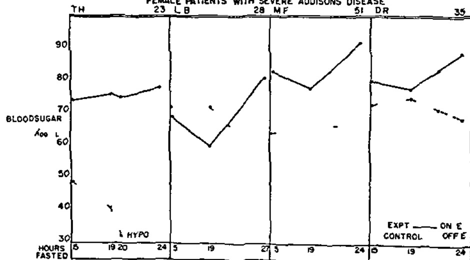


FIGURE 43

FURTHER CLINICAL STUDIES WITH ACTH AND ADRENAL CORTICAL HORMONES

GEORGE W THORN
Department of Medicine Harvard Medical School

A DISTINCTION should be made between the clinical use of ACTH and cortisone in pituitary and adrenal cortical deficiency as contrasted with the use of these hormones in diseases unaccompanied by detectable abnormalities in adrenal secretion In the presence of adrenal cortical insufficiency correction of the well established metabolic deficiencies constitutes as essential prerequisite for clinical recovery In contrast the typical metabolic changes attributable to these hormones may lead to undesirable complications when they are administered in large quantities to patients with nonendocrine diseases It appears that many of the benefits obtained in this latter group of patients are not primarily metabolic but are due to an action of the adrenal steroids which might be termed anti inflammatory or anti allergic Dr Dougherty and Dr Opsahl have discussed important aspects of this problem Our own observations would certainly confirm those of Dr Opsahl in the demonstration that ACTH and cortisone inhibit hyaluronidase activity (See Figure 40 page 144)

I should like to present an outline of the four phases of our clinical studies on ACTH and cortisone which may be used as a basis for discussion by this group

I EXPERIENCE WITH THE USE OF CORTISONE IN DISTURBANCES OF ADRENAL FUNCTION (1 2)

Treatment of Addison s Disease

The administration of cortisone in doses ranging from 12 5 mg to 25 mg of the saline suspension injected intramuscularly once daily has resulted in the improvement of the metabolic defects which persist in Addisonian patients maintained on desoxycorticosterone alone The three most important defects in the desoxycorticosterone treated Addisonian which serve as indications for supplementary cortisone are first hypoglycemic manifestations second a positive Robinson Kepler Power water test suggesting excessive antidiuretic activity and third electroencephalographic changes characterized by a slowing of the

ably except in patients with Addison s disease but it does modify insulin sensitivity particularly *in respect to the symptomatic response* to hypoglycemia there is an increase in glycogen reserve and an increased threshold of the nervous system of hypoglycemic manifestations

Ralli You can also condition the hypoglycemic reaction of an animal by hydration

Conn I think that Dr Thorn s point on which I will also show some data in a few minutes is correct If you test the effect of maintenance doses of a carbohydrate active steroid in the Addisonian you can demonstrate the effect of that steroid in preventing the hypoglycemia of fasting much more readily than you can show resistance to insulin

Thorn Twenty five milligrams of cortisone daily restores the capacity of a patient with Addison s disease to fast quite satisfactorily Now if the dose is increased to 100 mg per day for an unlimited period and with an unlimited dietary intake a much greater increase in glycogen reserves and further improvement in the capacity of the nervous system to withstand hypoglycemia are obtained The final

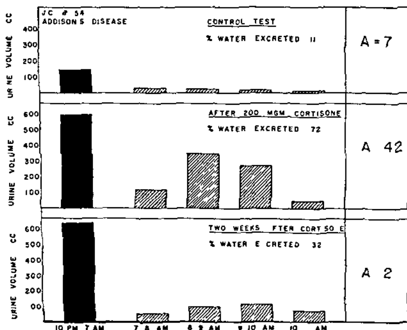


FIGURE 45

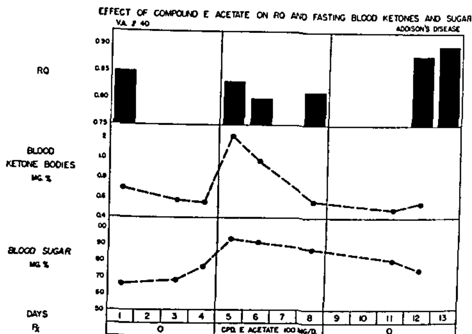


Figure 41

mobilize glycogen rapidly (glycogenolysis) This is demonstrated by the hypoglycemia which may occur after intravenous insulin administration despite cortisone therapy On the other hand if very large doses of cortisone are given in conjunction with a high carbohydrate intake it is possible to increase the glycogen desposits to such an extent that the hypoglycemic effect of insulin is buffered substantially

Astwood Can that actually be shown or are you just supposing it to be the case?

Thorn We have shown that the insulin reaction in patients with Addison s disease is reduced considerably by cortisone therapy but that insulin hypoglycemia is not prevented Two factors need to be considered a) the actual decrease in blood sugar levels and b) the patient s nervous system reaction to hypoglycemia In earlier studies we have demonstrated that adrenal extract prevents hypoglycemic manifestations at a blood sugar level at which they may be observed in the adrenalectomized untreated animal It is disappointing that even with cortisone insulin sensitivity persists

Loeb Regardless of dosage?

Thorn At the usual maintenance dose for Addisonian patients i e 12 5 to 25 mg cortisone daily

Cortisone therapy does not increase the fasting glucose level appreci

or more daily by intramuscular injection oral tablets or implanted pellets four showed complete reversal of the water test to normal and three showed marked improvement toward normal whereas two showed no change Neither of the two patients who failed to show a significant change was treated with doses of cortisone larger than 10 mg daily

Sayers Was the patient on desoxycorticosterone?

Thorn All were maintained on desoxycorticosterone At a 10 mg dose level of cortisone one does not in most instances obtain a normal water test At a 25 mg dose level of cortisone we do observe a complete reversal of the water test unless there is complicating hypothyroidism renal or hepatic disease

CORRECTION OF ABNORMALITIES IN ELECTROENCEPHALOGRAM AND ELECTROCARDIOGRAM Improvement in the electroencephalogram may be observed within a few days after the administration of 50 to 100 mg of cortisone (Figure 46) In the range of physiological replacement therapy i e 12 5 to 25 mg daily maximal improvement in electroencephalogram and electrocardiogram will be observed in one to three months A summary of the changes in electroencephalogram in

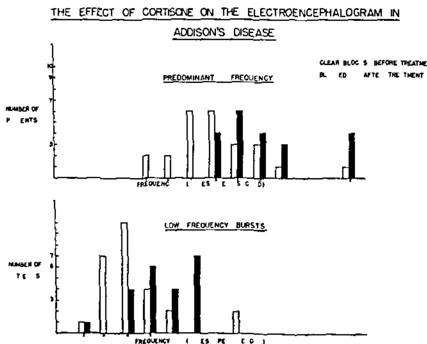


FIGURE 47

point which I would like to emphasize is that desoxycorticosterone does not prevent fasting hypoglycemia in a patient with Addison's disease except insofar as over all improvement in appetite and increased food intake reduce the hypoglycemia. Cortisone does protect the patient. Compounds A and B do in larger dosages.

Pincus: Twelve to 25 mg. is the dose for the Addisonian?

Thorn: Twelve to 25 mg. is the maintenance dose in a patient with Addison's disease. In our earlier studies 100 mg. of cortisone a day for four or five days was employed.

CORRECTION OF IMPAIRED WATER TOLERANCE. The apparent inhibitory effect which cortisone exerts on the excessive antidiuretic activity observed in patients with Addison's disease is illustrated in Figure 45. Prior to cortisone therapy there was a high level of antidiuretic activity in the urine and an impaired ability to excrete a water load; following cortisone therapy these defects in water metabolism have been improved. Our experience in this regard may be summarized as follows. Of nine patients who received cortisone in doses of 10 mg.

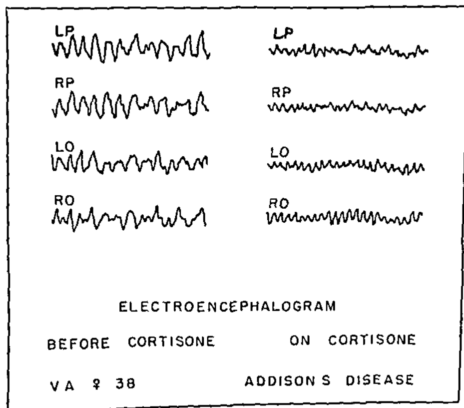


FIGURE 46

unique in this particular effect, have you tried any of the other corticosteroids?

Thorn No so far we have tried only cortisone We were quite encouraged to observe the rapid changes in the electroencephalogram which did occur with this hormone It would not be difficult to screen other steroids if their action approaches cortisone

Conn We have been able to produce a change of the same kind with compound B

Thorn That is very important One can be reasonably certain of restoring the electroencephalogram of a patient with Addison s disease to normal with doses of 25 mg of cortisone daily for a period of several weeks

Selye With what amounts of DCA have you compared this effect? May the effect be a matter of dosage?

Thorn It is important to decide whether we are working at the physiological level of hormone concentration In animals you can give unlimited quantities of desoxycorticosterone In man of course this is not feasible unless it is administered in a rapidly excreted form such as the glucoside of desoxycorticosterone We did not give it for a prolonged period

Sayers Dr Woodbury has some work which bears on this problem First in connection with the blood glucose level studies in rats agree with your conclusion Dr Thorn The level of blood glucose does not have much influence on brain excitability It should be pointed out that Dr Woodbury is measuring brain excitability by electroshock threshold which may very well be a different aspect of brain function than EEG In the adrenalectomized animal there is a considerable increase in brain excitability as measured by electroshock threshold The change in threshold is not due to a decrease in the concentration of blood sugar DCA decreases brain excitability whereas cortisone increases brain excitability

Thorn That is an interesting point The Addisonian patients show the classical changes in the electroencephalogram before any treatment at all We cannot state whether desoxycorticosterone aggravates some of these changes Certainly it appears that such may be the case in relation to certain electrocardiographic changes where one is dealing primarily with Na K ratios

Pincus I think the significance of the effect on the alpha rhythm particularly is something that should be kept in mind in contrast to for example the anesthetic effect Judging by a rather small amount of data in the literature Dr Hoagland has come to the conclusion there must be a dissociation between the two Quastel s work shows a parallelism between anesthetic effect and inhibition of certain parts of

patients with Addison's disease treated with cortisone is presented in Figure 47 Hypothyroidism limits this beneficial action of cortisone and adrenal cortical insufficiency untreated with cortisone, limits the effectiveness of thyroid hormone therapy in this same respect The results in the figure were from studies on a patient with probable primary thyroid deficiency and Addison's disease The adrenal deficiency was primary since she received no stimulation when continuous ACTH treatment was given We were not able to test her response to TSH We did observe that large doses of cortisone failed to correct the abnormality of the electroencephalogram until supplementary thyroid administration was added

Loewi In your opinion, is the recovery of the electroencephalogram following cortisone in Addison's disease due to the rise of the blood sugar? It has been frequently shown—for the first time to my knowledge by Bremer—that a low blood sugar impairs the electroencephalogram and that injection of glucose restores it to normal I am sure you have considered the first possibility?

Thorn In 1942 in conjunction with our studies with Hoffman at Johns Hopkins we showed that glucose did not restore the electroencephalogram in patients with Addison's disease nor did the administration of adrenal cortical extract in the quantity of 10 to 20 ml daily The abnormality in the electroencephalogram in the patient with Addison's disease is one of the most persistent defects that one observes and it requires continued cortisone administration at fairly adequate levels to restore the electroencephalogram to normal We do not feel that the changes in the electroencephalogram are due to the slight increase in blood sugar levels observed during cortisone therapy

Conn May I interject a remark at this point? In a patient with combined Addison's disease and diabetes whom we have studied recently and in whom the blood sugar was abnormally high the same findings that Dr Thorn has shown were present Thus these abnormal waves are present in Addison's disease even in the presence of hyperglycemia

Loewi This of course is a different story as the diabetic does not utilize the sugar in a normal way

Thorn There is no evidence that the brain in the diabetic does not utilize glucose is there?

Conn No

Long A question mark on that one too That statement is based on measurements on the respiratory quotient

Pincus Have you found whether any other steroids would affect this? I know DCA does not In view of Gordon's work on the effects of various steroids on brain respiration showing that cortisone is not

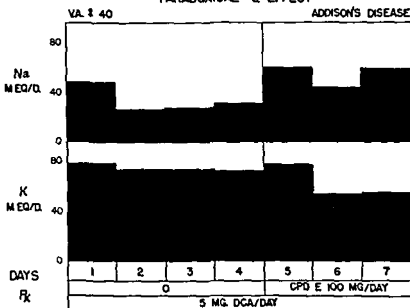


FIGURE 48

Loeb When you say the patient cannot be maintained on cortisone alone at what level of sodium intake do you mean?

Thorn About 6 to 8 gm total

Loeb It seems a little funny because you can maintain many Addisonians on just salt alone without cortisone It seems queer that if you take both adrenals out of a man with normal kidneys that on a diet containing 6 to 8 gm of salt he will lose salt rapidly on 50 mg of cortisone a day

Thorn I am certain that patients could be kept in balance with cortisone alone if large quantities of supplementary salt were also administered As I see it an important point in our discussion is whether or not cortisone alone in an optimal dose i e 25 mg will allow a patient to maintain a salt balance on a diet of his own selection If this is not true in the bilaterally adrenalectomized patient and we have shown that it is not true then we must assume that a hormone or hormones other than 25 mg of cortisone are normally being secreted by the adrenal

Long Dr Thorn you said if you give 50 mg of cortisone a day and a restricted salt intake the serum sodium steadily falls?

Thorn This was not salt restricted intake This was 6 or 8 gm daily He had 3 gm of supplementary salt and about 4 or 5 gm of diet salt

the respiratory system of the brain (3) This also is paralleled by Gordan s work (4) whereas on the frequency there is no parallelism

Selye I cannot remember what Carl Hartman found when he studied the EEG *in rats anesthetized with steroids Do you remember that?*

Pincus Hoagland has done some work on adrenalectomized rats brain wave frequencies and finds that DCA does not restore the fre quency drop, whereas adrenal extract (ACE) does and this does not parallel the effects on brain respiration (5) Anesthesia and respiration in brain seem to go hand in hand whereas the effects on electrical activity and anesthesia do not go hand in hand

Thorn Somerville (6) has studied the effect of cortisone on the abnormal electrocardiogram characteristic of patients with Addison s disease He has shown that cortisone administered in doses of 163 to 1500 mg over periods of five to two hundred and thirty days to twelve patients with Addison s disease resulted in improvement in ten cases and no change in two The improvement was represented by a change of T waves from an inverted or flat form to upright In one case a prolonged P R interval was shortened and in six a prolonged Q T interval reverted to normal All of our patients receiving large doses of cortisone are also given potassium chloride as supplementary therapy If we did not give the potassium supplement we would get striking changes in the electrocardiogram

White Does this occur in the normal?

Thorn Yes

White Does the non Addisonian given ACTH show changes with out potassium?

Thorn Yes without the potassium supplement changes in the electrocardiogram characteristic of potassium deficiency may occur

ELECTROLYTE BALANCE It is impossible to obtain a satisfactory sodium and chloride balance in the majority of patients with Addison s disease treated with cortisone alone in a dosage which does not induce hypercorticism It is quite possible however that patients might be regulated well with large doses of sodium chloride by mouth and cortisone as the only adrenal steroid As a matter of fact the mainte nance dose of desoxycorticosterone acetate may need to be increased appreciably to maintain a good sodium chloride balance when 12 5 to 25 mg of cortisone is used (Figure 48) When cortisone therapy is added to a basic maintenance dose of desoxycorticosterone one may observe increased sodium excretion temporarily rather than an addi tive sodium retaining effect This action suggests an antagonistic effect of these two steroids We have demonstrated that following bilateral complete adrenalectomy in man 50 to 100 mg of cortisone daily did not prevent the loss of large quantities of sodium in the urine

myself because you know I still belong in the group of die hards that thinks maybe there is some other hormone like DCA in the adrenal

Thorn Let us get the facts straight We have done the same thing with patients with Addison s disease given 100 mg of cortisone daily However patients with Addison s disease cannot tolerate 100 mg of cortisone indefinitely and when one reduces the dose of cortisone to a maintenance level i e 12 5 to 25 mg daily, one does obtain sufficient salt retention to permit most patients to select their diet *ad lib*

Long How important is the level of sodium in the serum? It appears to be much more critical in the human than in the dog because the experience of Dr Loeb and his colleagues reminds me of an experiment carried out a good many years ago by Swingle who totally adrenalectomized dogs and when the sodium fell to the characteristic low level gave them nothing but cortical extract and a diet low in sodium They lived over a year in good health with a low serum sodium

Loeb I think it is very possible those animals had a restoration of blood volume without corresponding increase in sodium I would guess the contraction of the blood volume would be more important than the actual serum sodium level

Ingle It is possible to maintain adrenalectomized dogs and rats indefinitely on cortisone alone The dosage required is relatively higher than is being used for the treatment of patients with Addison s disease

Thorn Are you able to support a normal growth curve?

Ingle No

Thorn In the case of dogs you can keep them alive with cortisone You cannot get the normal growth curve that you get with salt alone or desoxycorticosterone The important point now is that when cortisone is given to an animal or patient who is already receiving desoxycorticosterone the situation may be somewhat different from giving the same dose of cortisone to a patient or animal with the adrenals and without desoxycorticosterone

Selye What happens if you give DCA to a patient with Cushing s disease?

Thorne *As you know Dr Soffer has reported the ineffectiveness of* desoxycorticosterone in patients with Cushing s disease and we have been able to substantiate this We have given 40 mg of desoxycorticosterone a day to a patient with Cushing s disease without causing any appreciable salt retention

ORAL ADMINISTRATION OF CORTISONE The observation that the oral administration of cortisone is an effective method of therapy in patients with Addison s disease has great clinical importance It is also apparent that this anti inflammatory and antiallergic effects of cortisone may be obtained with orally administered hormone Most sur

Long What may I ask was the extent of those falls? How long did they go on?

Thorn I cannot give you the changes in serum sodium but the excretion of sodium in the urine amounted to 150 mEq a day We did not let him progress further because of cramps and decreased plasma volume

Sayers Dr Astwood what dose of cortisone did you use to maintain a positive sodium balance?

Astwood We have three patients under treatment who receive 10 15 and 35 mg a day respectively

Sayers You did maintain them in positive sodium balance?

Astwood Not on the basis of balance studies but they have done well

Conn What is the salt intake?

Astwood Diet salt, no added salt

Thorn I might relate an experience we had this past summer One of our patients who was sensitive to desoxycorticosterone—1 or 2 mg caused edema and it is necessary for her to restrict her salt intake while being maintained on desoxycorticosterone—she was given 25 mg of cortisone in addition to 1 mg of desoxycorticosterone In September she came into the hospital in extreme dehydration having lost large quantities of sodium chloride Serum sodium was down BUN was elevated and the hematocrit showed hemoconcentration It appeared to us that this patient did not exhibit the usual salt retention on desoxy corticosterone which she had experienced previous to the cortisone

Ralli You stated earlier that cortisone was a salt retaining hormone It appears that this statement cannot be made without qualification

Thorn Certainly you can make it without qualification Cortisone is a salt retaining hormone However the salt retaining effect is not as great as that of desoxycorticosterone

Loeb Dr Thorn it might be of interest in relation to this to point out that George Perera and Abbie Knowlton had an Addisonian who was maintained on 16 gm of salt alone There were 6 gm of salt in the diet and 10 gm a day added They cut out the added salt of sodium chloride and the patient went into classical crisis in one or two days with a drop of serum sodium to about 131 mEq It was sufficiently alarming so that they wondered whether they would dare use cortisone alone or not They nevertheless treated that patient solely by the ad ministration of oral cortisone They gave 200 mg the first day 150 mg the second then smaller doses and the patient made a complete recovery without an infusion and without any DCA I merely say there is one instance where the total intake was 6 gm and with cor tisone alone a restoration was brought about I am talking agin

the increased urinary excretion of corticosteroids is very prompt

Thorn It is but there is no change in the distribution as far as the 17 ketosteroids

Pincus That is what has surprised us most but on the basis of fractionating cortical steroids the same type of excretion is not observed after intramuscular injection

Sayers What about the quantitative aspects the amount of material that is excreted in the urine by intravenous as compared with oral administration?

Pincus We have not undertaken the intravenous injection of cortisone We have compared intramuscular with oral administration (7) The quantity excreted is somewhat greater after oral administration We think that may be a little deceiving because as Dr Thorn s data indicate the intramuscular material may be retained and absorbed more slowly

Long Were you surprised that cortisone was active by mouth?

Thorn Very

Long I think Dr Ingle in 1934 indicated the activity of these steroids by mouth

Thorn That was in the rat Dr Grollman showed this years ago The rat has always done well with adrenal extract given by mouth but with patients we were never able to maintain them except with extremely large doses We have given as much as 40 to 50 ml of extract a day by mouth

Replacement Therapy in Patients with Hypertension Subjected to Bilateral Complete Adrenalectomy

The important role which the adrenal cortex plays in the maintenance of hypertension has been investigated experimentally and to a limited extent clinically The beneficial effect of adrenal resection in correcting the hypertension of patients with Cushing s disease is well established * Further evidences for hypertensive factors secreted by the adrenal cortex are to be found in the experiments of Haynes *et al* (8) who observed an appreciable increase in hypertensinogen in the blood of dogs treated with ACTH and in the study of Dexter *et al* † who observed a marked increase in peripheral arteriolar resistance in a patient with rheumatoid arthritis treated with ACTH

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* Personal communication from Dr Randolph Sprague

† Personal communication

prising is the fact that cortisone administered orally appears to be as effective as cortisone administered intramuscularly The immediate eosinopenia induced by orally administered cortisone is much greater and occurs much more rapidly than that following intramuscular injection and approximates that observed following intravenously administered hormone Using the eosinophil fall as an indication of duration of effect, it can be shown that 25 to 50 mg of cortisone by mouth either in tablet form or as a suspension, induces an eosinopenia which attains a maximal value in four to six hours At the end of eight hours a definite escape has occurred When 100 mg of cortisone is administered as a single oral dose escape does not begin to occur until eight to ten hours In some instances 25 mg every six hours is effective but this dose cannot be depended upon to produce a satisfactory eosinopenia (Figure 49)

Pincus We have treated ten patients with oral cortisone and have observed rapid early changes in the various measurements made Even

COMPARISON OF EFFECTIVENESS OF CORTISONE BY VARIOUS ROUTES

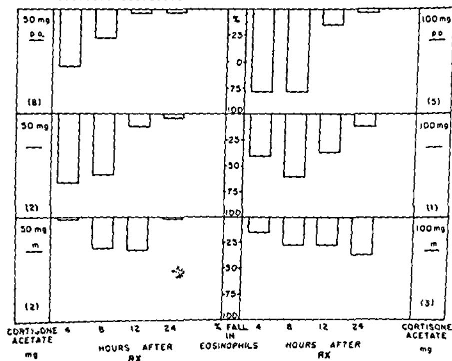


Figure 49

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* P o l mm n cation f m D R ndolpl Sp g

† P o l communic t n.

With the advent of cortisone it has become possible to investigate the role of the adrenal cortical hormones in the maintenance of malignant hypertension Since our studies have shown little or no increase in adrenal activity in these patients, it is obvious that the role played by the adrenal hormones is a supporting one rather than a causative one Such a concept suggests that complete, or near complete, removal of both adrenals might be necessary to attain a successful fall in blood pressure in most patients with essential or malignant hypertension The possibility of accomplishing this same end by antagonizing desoxy like adrenal steroids with large doses of cortisone has been investigated by Perera and his group (9) Although some fall in blood pressure has been observed in these patients it has not been possible to date to restore blood pressure to normal levels by this means and in addition some evidences of excessive cortisone therapy have limited the procedure Green and his colleagues have reported the successful removal of nearly all adrenal cortical tissue in a patient with hypertension and diabetes (10) This patient has been maintained in good condition with adrenal cortical extracts and the blood pressure has attained essentially normal levels Lukens (11) in Philadelphia has recently reported on the benefit to be derived from subtotal bilateral adrenalectomy in patients with malignant hypertension

We have felt that a total adrenalectomy might be the most desirable procedure both from a physiological and clinical point of view since it is always difficult to estimate accurately the amount of residual adrenal tissue following a generous subtotal resection and since regeneration of cortical tissue might complicate the interpretation of changes following operation During our two years experience in the treatment of patients with Addison s disease with cortisone combined with a minimal maintenance dose of desoxycorticosterone acetate we have observed almost complete rehabilitation of the majority of these patients Thus adrenal cortical insufficiency induced by complete total adrenalectomy did not appear to pose an insurmountable clinical problem It was hoped that the substitution of cortisone with minimum quantities of desoxycorticosterone following bilateral complete adrenalectomy might permit a hypertensive patient to lead a relatively normal life and permit blood pressure to attain a normal value

Indication that this might be the case was obtained from a study on a patient with Addison s disease who was known to have had severe hypertension with marked retinal changes for a period of ten years prior to the development of Addison s disease With the onset of acute adrenal crisis his blood pressure fell to normal levels but the patient was completely incapacitated With desoxycorticosterone substitution the patient was partially rehabilitated but hypertensive levels

of blood pressure again recurred With a minimum maintenance dose of desoxycorticosterone and supplementary cortisone the blood pressure fell to normal levels and the patient was able to return to professional activity for the first time in over two years (Figure 50)

Ralli What happened to the retinal changes?

Thorn He is totally blind in the right eye He has had no further evidence of hemorrhage or exudates in the left eye

Selye Is not that somewhat in contradiction with the view that the corticoids are only the sustaining factor?

Thorn Yes I think there is evidence to suggest that desoxycortico sterone may have a vascular effect However we are not certain yet whether the normal adrenal secretes desoxycorticosterone

Complete evaluation of total bilateral adrenalectomy as a means of regulating hypertension must include consideration of the possible role of the adrenal medulla The well known effect of epinephrine and particularly nor epinephrine as a vasoconstricting agent and the wide

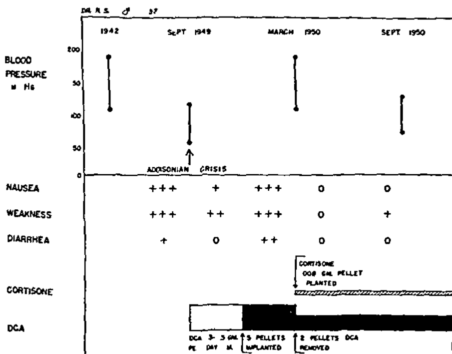


Figure 50

fluctuations in blood pressure which may follow stimulation of the adrenal medulla suggest that the removal of a substantial quantity of chromaffin tissue such as would occur in bilateral complete adrenalectomy, might prove of additional benefit in the regulation of hypertensive patients

With all of these considerations in mind, and with an available supply of cortisone we have undertaken a study of the effect of bilateral complete adrenalectomy on hypertensive patients whose course appeared rapidly progressive and whose clinical state was such that sympathectomy had been rejected and the rice diet had proved ineffective

The first patient was successfully operated upon in the spring of 1950 in a two stage complete adrenalectomy but unfortunately died eight days following the second stage of the operation as a result of a coronary occlusion The patient s reaction to operation had been perfectly satisfactory and it was felt that his demise represented a risk which had to be faced in this group of patients with advanced vascular disease The efficacy of substitutive hormone therapy however was well demonstrated during and immediately following the operation

A second patient had his two stage operation in the summer and early fall of 1950 and his clinical course has been remarkably gratifying up to this point Of particular interest is the fact that following operation this patient lost large quantities of sodium chloride despite the administration of 100 mg of cortisone daily Salt balance was subsequently established with a minimum maintenance dose of 25 mg of cortisone by mouth daily and a supplement of 2 5 mg of desoxycorticosterone once or twice weekly This patient s symptoms of hypertension completely disappeared and he has resumed work as a hospital orderly for the first time in two years Not only has his heart size returned to normal but his renal function appears to have improved appreciably despite the fall in blood pressure to near normal levels (Figure 51) The patient s physical condition is excellent and his blood volume is almost identical with that observed preoperatively Prior to adrenalectomy this patient had been studied for a prolonged period in the hospital under rest sedation a diet of minimum salt intake and finally a rice diet the latter being very poorly tolerated and ineffective

Ralli The pressure runs 158 for diastolic and a little less than 200 for the systolic?

Thorn That is right On the rice diet this is a basal blood pressure taken in the morning after a night s rest before the patient is allowed out of bed

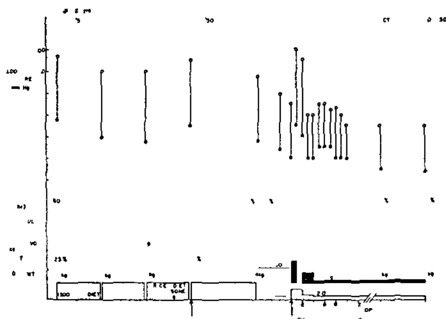


FIGURE 51

Ralli Do you think that those levels of blood pressure constitute a vehement form of hypertension?

Thorn Yes I think at complete rest with a low salt diet this was a disabling form of hypertension for this patient This is obviously not the most severe form of hypertension that we have seen but it was incapacitating for this patient Furthermore his disease was advanced to the point where he was refused a sympathectomy I do not think it is wise to discuss the problem of hypertension at this time with the few cases that we have studied I believe that we shall obtain some interesting physiological observations on man following bilateral complete adrenalectomy and that some relationship with hypertensive vascular disease may ultimately become manifest

Loeb What was the renal status in this patient?

Thorn He had a glomerular filtration of 132 ml per minute

Sayers What about the paper which just appeared in the *J A M A* by D M Green concerning bilateral adrenalectomy in a patient with essential hypertension (10) Do your studies confirm Green?

Thorn That patient had both diabetes and hypertension and a relatively complete adrenalectomy was performed The patient has done

well over a two year period on extract We have successfully adrenal ectomized a third patient At the present time this patient has recovered well from his operation has a normal blood pressure and is being maintained on 25 mg of cortisone daily On this dose of cortisone there has been a negative sodium chloride balance and a steady loss in weight but since this patient had edema and marked cardiac enlargement prior to operation it has been felt desirable not to add any supplementary desoxycorticosterone to date There has been no reduction in plasma volume and no evidence of postural hypotension

It is not proposed that total bilateral adrenalectomy be considered at this time as a method of treatment for patients with hypertension It is obvious however that careful studies of the cardiovascular changes which follow bilateral complete adrenalectomy may provide much useful information in unraveling certain important aspects of the hypertensive problem We believe that in most instances the adrenal cortex is not responsible for hypertension but that it is impossible to *maintain hypertension in the absence of the adrenal cortex* It would appear that the substitution of cortisone for normal adrenal cortical and medullary function may permit the satisfactory maintenance of normotension in a previously hypertensive patient It may be necessary to administer desoxycorticosterone from time to time to prevent excessive sodium chloride loss in such patients

Astwood Do you think it would be of interest to compare the effect of adrenal demedullation?

Thorn I do We cannot eliminate the role played by the adrenal medulla in our studies In addition to the effect of the adrenal medulla it is interesting to study the effect of prolonged rice diet on adrenal cortical function We have some evidence that a prolonged dietary deficiency of this type does reduce adrenal activity as evidenced by the 17 ketosteroid excretion and the refractoriness of the adrenal to ACTH

Dougherty This is on the rice diet?

Thorn Yes On a prolonged rigid rice dietary regimen there was evidence of decreased adrenal cortical functions

Rall Were the adrenals of this patient examined microscopically?

Thorn The adrenals were normal microscopically

Selye What did the kidneys look like?

Thorn Renal biopsy on these two patients showed arteriosclerosis in both cases fibrosis of occasional glomeruli in one and fibrosis of 20 to 50 per cent of glomeruli in the other

Use of Cortisone in the Amelioration of Adrenal Virilism

If one upholds the theory that more than a single hormone is secreted by the adrenal cortex then the possibility of using cortisone to

TABLE XI

Hirsutism Oligomenorrhea Obesity

P O F 38

	17 ketosteroids mg/24 hr
Cortisone acetate	
Control	15 3
48 hour ACTH	13 1
12 5 mg b i d	
(60 days)	3 2*

Menses returned

induce inhibition of the anterior pituitary secretion of ACTH and subsequent atrophy of the adrenal cortex presents itself By this means it might be possible to decrease the secretion of adrenal androgenic substances Dr Lawson Wilkins in Baltimore was the first to report success administering small doses of cortisone and obtaining a marked reduction in the 17 ketosteroid output in children suffering from viril ism (12) Our own studies support this observation In a patient with adrenal virilism a partial adrenal resection carried out several years ago failed to modify the hirsutism This patient when treated with 12 5 mg of cortisone by mouth twice daily demonstrated a remarkable fall in 17 ketosteroids This was associated with clinical improvement (Table XI) The possibility of inhibiting the secretion of adrenal steroids other than 11 17 oxysteroids by the use of relatively small doses of cortisone would appear to be feasible

It has been demonstrated over and over again that patients with rheumatoid arthritis or other chronic diseases undergoing prolonged therapy with cortisone have shown marked inactivation of the adrenals at the conclusion of the period of cortisone treatment ACTH given at the conclusion of cortisone treatment will often not produce an appre ciable fall in eosinophils or a rise in 17 ketosteroids or 11 oxysteroids

Bauer We have some studies which show that you do get the same ACTH response

Thorn There is the possibility that with very large doses of cortisone the inhibition of the adrenal may be local as well as at the pituitary level At least it has seemed suggested in some of our studies that the delay in responsiveness to ACTH following cortisone in large doses was much prolonged This would probably not be the case if ACTH suppression were the only factor since we have studied a large number of Dr Cushing s patients with X ray to the pituitary and removal of

the gland whose adrenals are activated fairly rapidly with ACTH I would like to ask Dr Long if one removed the pituitary of an individual, would you expect a good response from ACTH ten to twenty days after the hypophysectomy?

Long No

Thorn Next we come to

II THYROID ADRENAL RELATIONSHIPS

During the past two years, with the use of ACTH cortisone and purified thyroid stimulating hormone, it has been possible to elucidate a number of complex interrelationships which exist between the thyroid and adrenal glands I should like to discuss very briefly three of these which have a direct relationship to ACTH and cortisone administration

A The limitation in adrenal response to ACTH which occurs in states of hypothyroidism

B The depression in thyroid activity which occurs with long continued ACTH or cortisone therapy

C The calorigenic effect of cortisone which appears to be independent of thyroid gland activity

The Limitation in Adrenal Response to ACTH Which Occurs in States of Hypothyroidism

Patients with myxedema very frequently fail to show a fall in eosinophils when given the standard dose of ACTH In this respect the response is identical with that seen in Addison s disease The refractoriness to ACTH stimulation is not limited to the eosinophil response but may also be observed as an impaired 17 ketosteroid response in the standard forty eight hour ACTH test However when such patients are treated with thyroid hormone over a period of one to two months normal adrenal response may be elicited with ACTH (Figure 52)

The following study suggests that the improvement in the electroencephalogram observed in patients with Addison s disease treated with cortisone is also dependent upon an adequate quantity of circulating thyroid hormone In a patient with marked hypothyroidism and Addison s disease with profound alterations in the electroencephalogram the administration of cortisone in doses as large as 300 mg daily produced only minimal improvement in the electroencephalographic pattern The administration of thyroid in the absence of cortisone therapy also failed to produce striking improvement whereas combined therapy with cortisone and thyroid extract resulted in a rapid reversal of all abnormalities in the electroencephalographic tracing

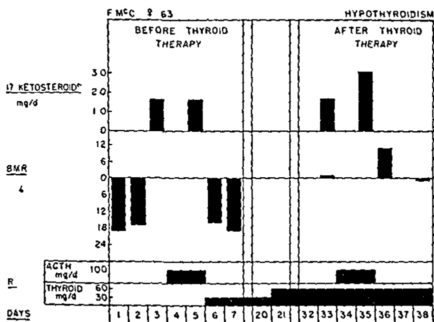


FIGURE 52

The Depression in Thyroid Activity which Occurs with Long continued ACTH or Cortisone Therapy

In general the continued administration of ACTH or cortisone will ultimately induce a depression of thyroid function although in many instances there is an initial transient increase in the rate at which the thyroid gland accumulates radioactive iodine (13) Occasionally patients with hyperthyroidism treated with ACTH after a period of careful standardization have shown a satisfactory fall in basal metabolic rate protein bound iodine in the serum and the rate at which radio active iodine accumulates in the gland It seems possible that the reduction in thyroid activity might reflect in part at least a depressing effect of the high level of adrenal steroids on anterior pituitary activity This hypothesis was supported by an experiment in which it was shown that the administration of thyrotropic hormone to a patient whose thyroid activity had been reduced following ACTH therapy resulted in a marked increase in the rate at which radioactive iodine was picked up by the thyroid gland an elevation of the basal metabolic rate and an increase in protein bound iodine (Figure 53) It is possible

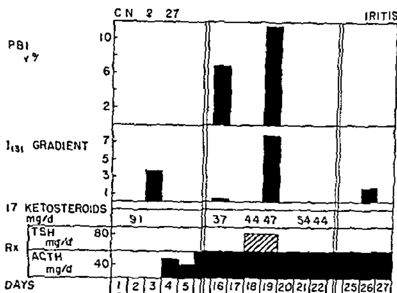


Figure 53

that a reduced response to ACTH administration may occur as a result of suppression of thyroid activity secondary to the high level of adrenal steroids Recently this theoretical concept has received clinical confirmation in observations in which the administration of thyroid seemed to restore the sensitivity of patients to ACTH

The Calorigenic Effect of Cortisone Which Appears to be Independent of Thyroid Gland Activity

In athyreotic patients on a constant dose of desiccated thyroid ACTH or cortisone induce a slight but definite increase in the basal metabolic rate without a change in serum protein bound iodine level (13) This calorigenic action of ACTH and cortisone may account for the fact that patients on long continued adrenal hormone therapy exhibit a normal basal metabolic rate although thyroid activity may be suppressed

III CLINICAL STUDIES ON REACTION TO STRESS

Major Surgery

A fall in the level of circulating eosinophils occurs regularly in

patients subjected to major surgery in whom adrenal function is normal *Within a matter of five to ten hours following a major operation the* eosinophils practically disappear from the blood (Figure 54) and the level remains low for a period of two to three days after which there is a rebound eosinophilia The presence of impaired adrenal functional reserve may be suspected if a normal level of circulating eosinophils is observed in the blood within the first forty eight hours following surgery

When the adrenal cortical response is tested postoperatively by administering 25 mg of ACTH during the convalescent period one observes a normal response i e a fall of over 60 per cent in nearly all patients whereas the response to epinephrine (0 3 mg) may be impaired in an appreciable percentage of patients This dissociation suggests that during the convalescent period the adrenal cortex is storing hormone and that the failure to discharge large quantities of hormone at this time probably reflects an altered responsiveness in the hypothalamic pituitary initiation of ACTH secretion

The contributing role played by the adrenal steroids in the classical

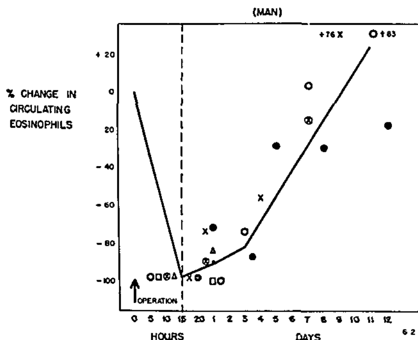


Figure 54

reaction to surgery has been studied by Dr J S L Browne in Montreal and Dr Francis D Moore in Boston Dr Moore has demonstrated that when a normal control subject was exposed to all of the situations surrounding a major operation including hospitalization semistarvation parenteral fluid therapy, etc, but without anesthesia or actual surgery, and when in addition the subject was given ACTH the metabolic changes which were recorded under these circumstances simulated very closely those of a patient actually undergoing major surgery (14) When the experiment was done with ACTH there still remained one outstanding metabolic difference in comparison with the observed metabolism of surgical convalescence This relates to the fact that with the ACTH dummy operation sodium diuresis commenced on the fourth day upon cessation of hormonal stimulus, whereas in normal surgical convalescence sodium diuresis is rarely observed prior to the tenth or twelfth day When the identical experiment was done without ACTH a metabolic response was produced which differed from surgery largely with respect to sodium and potassium metabolism There was also less tendency to retain sodium and much less tendency to excrete potassium than is seen after a major surgical procedure

It is becoming increasingly apparent that the nature of the response to ACTH and cortisone may be modified significantly by the initial state of the patient This may under certain circumstances be so marked as to give rise to a negative over all nitrogen balance in one instance and a positive nitrogen balance in another The factors responsible for modifying the action of adrenal hormones need to be elucidated

Burns

Several special considerations arise in the relation of burns to adrenal cortical function It appears likely that local products derived from the burn area may act as stimulating agents to the hypothalamic pituitary adrenal system In addition there is a growing body of evidence that the metabolic response observed following burns is in part independent of the adrenal cortex There is the added problem however that toxic substances derived from the burn area may injure the adrenal cortex directly Hemorrhage and parenchymatous changes within the adrenal gland have been observed frequently in seriously burned patients

In relation to ACTH and cortisone therapy one must first consider whether or not adrenal insufficiency is present on the basis of adrenal injury or exhaustion If such a state is present ACTH would be contraindicated whereas large quantities of cortisone might be lifesaving If no evidence of adrenal cortical insufficiency is present then the possible benefits to be derived from an excess of adrenal cortical hormone

either produced by ACTH stimulation of the patient's gland or provided directly by the administration of cortisone might be investigated

There are several theoretical considerations which would suggest the usefulness of ACTH or cortisone in the treatment of seriously burned patients

1. The reaction of tissue surrounding the burn area might be greatly diminished and the absorption of toxic material retarded by mechanisms discussed previously
2. If hypotension were present adrenal cortical hormone action on liver and kidney might tend to support blood pressure by the effect of these substances on electrolyte balance and on the pressor and depressor mechanisms in the kidney and liver
3. Scar tissue formation might be inhibited whereas epithelization might be stimulated
4. A high circulating level of adrenal hormone might reduce the potential reaction to slight incompatibilities following blood and plasma transfusions and might improve the taking of homologous skin grafts

It is not possible at present to arrive at a definite conclusion regarding the usefulness of ACTH or cortisone in seriously burned patients It is apparent that the subject deserves extensive investigation particularly since in the seriously burned patient the phase characterized by Selye as one of adrenal exhaustion might play an important role in extending convalescence

Competitive Athletics

Another interesting field of investigation in relation to stress is a study of the mechanisms involved in a highly competitive fatiguing physical exercise illustrated by a four mile crew race Studies carried out by Renold *et al* (15) on members of a crew preparing for competitive racing may be summarized as follows During a practice race against time there was observed a marked fall in circulating eosinophils in all of the crew members During the subsequent intercollegiate competitive race although the time during which the course was covered was almost identical with that of the practice session it was noted that the eosinophils fell considerably more than during the practice session although obviously the physical work output was approximately the same The importance of anxiety as a factor causing adrenal cortical disturbance and subsequent eosinopenia in competitive athletes is well demonstrated by the fact that the coxswain and the coach showed an eosinophil fall almost identical with that exhibited by the crew members (Figure 55) The fact that severe physical stress alone failed to result in as marked a stimulation of pituitary adrenal function as did physical stress plus the stimulation of intercollegiate competition raises many interesting problems The fact that anxiety could induce

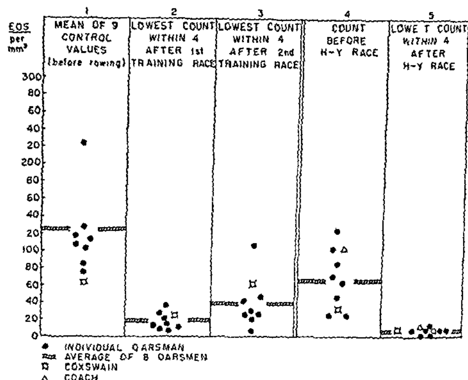


FIGURE 55

a fall in eosinophils of the same magnitude as that experienced by the crew members participating in the physical stress of the race indicates the large quantities of adrenal hormone which may be liberated during periods of mental and emotional stress These factors must be taken into account in considering the mechanism of adaptation of highly trained competitive athletes as well as the more widespread application in the adaptation of man to his environment

Dougherty You did not follow them for a period of time after?

Thorn We have a series of counts taken just before the race and at one four and twenty four hours thereafter The eosinophils were back to normal in twenty four hours In major surgery we obtained a similar curve Within six hours after a major operation there are few or no circulating eosinophils in the blood At the end of three days however the circulating eosinophils have obtained a normal or above normal value

Bauer Was it not Pincus who said that the event causing the most stress which a person leading a sedentary life has to face each day is

getting out of bed? This statement was made on the basis of eosinophil counts done on awakening and four hours later

Pincus That is right

Thorn The person with well marked anxiety may approach the eosinopenia observed in patients with Cushing s disease

Pincus We measured the 17 ketosteroids in students taking the Ph D examination There was a very marked increase in 17 keto steroid output following the examination about as great as any that we have observed

Dougherty The point just made has interesting implications Could the fact that a tense individual without expending muscular energy and yet exhibiting a fall in eosinophils be correlated with changes in the electroencephalogram related to hyperexcitability? Anxiety could increase the amount of hormone output but if this hormone is not utilized by muscular exercise it would accumulate and increase cerebral cortical activity

Thorn That is a very important point In almost every instance there are local or general conditions which would favor the utilization of the hormone which is secreted in response to stress But in the case of anxiety as far as we know there would not be the corresponding utilization of hormone

Dougherty I was wondering for instance in relation to treatment of anxiety states with electroshock insulin etc whether these stress stimuli are not increasing hormone utilization and thus probably lowering the cerebral cortical activity

Thorn Our last phase

IV USE OF ACTH AS A DEFINITE TEST FOR ADRENAL CORTICAL FUNCTION

A brief statement should be made about the present status of the use of ACTH as a measure of adrenal cortical function The four hour eosinophil response to a standard dose of ACTH (25 mg) appears to be a reliable index of reserve adrenal cortical function A fall of 70 per cent or more in the level of circulating eosinophils indicates a relatively normal adrenal cortical reserve The absolute level of circulating eosinophils may provide useful information since an eosinopenia in the absence of bone marrow inhibition would suggest an initial state of adrenal cortical hyperactivity whereas a normal or elevated eosinophil count in the absence of allergic manifestations would suggest a relatively inactive adrenal cortex The absence of a fall in eosinophils following the administration of a standard dose of ACTH does not

justify the diagnosis of Addison's disease, since temporary states of adrenal exhaustion or maximal output give a similar response It is important to appreciate the fact that severe depletion of body reserves as may occur in prolonged starvation in uremia, anorexia nervosa or ulcerative colitis may temporarily impair the responsiveness of the adrenal cortex to administered ACTH It does not appear that changes in the uric acid creatinine ratio and alterations in the excretion of other electrolytes add sufficiently to the eosinophil fall in the four hour screening procedure to justify their determination routinely

With any evidence of diminished functional capacity of the adrenal cortex or with persistence of clinical signs or symptoms of adrenal cortical hypofunction a forty eight hour ACTH test with measurement of changes in 17 ketosteroid excretion should be carried out (2) This is a more direct indication of adrenal cortical response It is customary to collect a standard twenty four hour urine specimen for 17 ketosteroid determination and then administer 25 mg of ACTH and continue with 10 mg of ACTH every six hours administering a total dose of 95 mg over a period of forty eight hours and measuring the 17 ketosteroid excretion from the twenty fourth to the forty eighth hour of ACTH treatment The eosinophils response to the initial injection of 25 mg of ACTH should also be noted, thus combining a four hour and forty eight hour test Obviously if 11 oxysteroids could be measured during the forty eight hour period of ACTH administration additional significant information could be obtained from the test Our experience in the use of the forty eight hour ACTH test as outlined above is summarized in Table XII

In addition to being able to measure the effectiveness of ACTH in stimulating the adrenals by studying changes in renal excretion of electrolytes and the concentration of sodium chloride and potassium

TABLE XII

Normal Males (7)			
4 hour eosinophil fall	63%	Control 17 ketosteroids	11 1
48 hour eosinophil fall	85%	48 hour 17 ketosteroids	22 9
		Mean rise 17 ketosteroids	11 4
Normal Females (6)			
4 hour eosinophil fall	83%	Control 17 keto teroids	6 3
48 hour eosinophil fall	95%	48 hour 17 ketosteroids	15 5
		Mean rise 17 ketosteroids	9 2

TABLE XIII

The Salivary Na K Ratio and Adrenal Cortical Activity

	Number of Patients	Average Salivary Na K Ratio
Normals	20	1 3 (0 24 2 1)
Addison s di ease untreated	5	5 0 (2 2 8 1)
Addison s disease treated	13	1 8 (0 9 2 52)
Cushing s disease	5	0 5 (0 26 1 8)

in the sweat it has recently been shown by Dr Thomas Frawley that following ACTH administration there is a reduction in the sodium potassium ratio in saliva (16) This provides a readily accessible means of studying changes in adrenal cortical function and should prove extremely useful in its clinical application (Table XIII)

Conn I have a little material on the metabolic effects of corticoster one (compound B) which I think might be interesting at this point Because of the limited availability of compound B very little work has been done with it in man Studies with compound B in rats have indicated that the substance produces effects not only upon the metab olism of electrolytes but also upon the metabolism of protein and carbohydrate The only studies of the effects of compound B in man of which I am aware are those reported ten years ago by Dr Thorn and by Dr Loeb and their respective associates

Dr Thorn had a total of 85 mg of compound B all of which he administered in a single day to a patient with Addison s disease He reported that the compound exerted effects upon electrolyte metabolism as well as upon the metabolism of carbohydrate and protein Dr Loeb s group spread its available 90 mg over a five day period in an Addison ian and reported no significant effects upon either electrolyte or organic metabolism Since that time no other reports on compound B in man have appeared

Through the courtesy of the Upjohn Company 10 gm of crystalline compound B in aqueous suspension was made available for our study Prolonged metabolic balance studies have been carried out upon two normal men and upon two cases of Addison s disease one with a co existing diabetes mellitus Observations upon two more cases of Addi son s disease have been made Each of the latter patients has received

justify the diagnosis of Addison's disease since temporary states of adrenal exhaustion or maximal output give a similar response It is important to appreciate the fact that severe depletion of body reserves as may occur in prolonged starvation, in uremia anorexia nervosa or ulcerative colitis may temporarily impair the responsiveness of the adrenal cortex to administered ACTH It does not appear that changes in the uric acid creatinine ratio and alterations in the excretion of other electrolytes add sufficiently to the eosinophil fall in the four hour screening procedure to justify their determination routinely

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48 hour eosinophil fall	95%	48 hour 17 ketosteroids	15 5
		Mean rise 17 ketosteroids	9 2

Conn One hundred milligrams a day for the first four days and 200 mg a day for the succeeding six days

Figure 57 compares in this same normal subject the relative effects upon urinary electrolytes of cortisone (compound E) corticosterone (compound B) and ACTH At a dose level of 200 mg per day compound B causes greater retention of sodium and chloride than does compound E Similarly the sodium and chloride diuresis upon cessation of these compounds is greater after compound B than after compound E On the other hand 100 mg per day of ACTH produces much more marked effects on renal excretion of electrolytes than is observed with either compound E or compound B at the dose level of 200 mg per day

Figure 58 shows the results obtained upon the second normal subject They are similar to those seen in the first normal subject but more pronounced. Increased renal excretion of potassium is observed during the administration of corticosterone

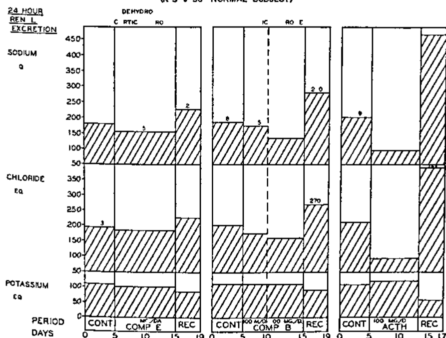


FIGURE 57

25 mg of compound B daily for sixty days A very rapid and remarkable return to normal health has occurred in the Addisonians receiving compound B

Figure 56 demonstrates the effect of large doses of compound B on renal excretion of electrolytes in a normal subject At a dosage level of 100 mg per day there occurred a sharp retention of sodium and chloride and when 200 mg per day were given a similar but more intense response occurred It is significant that escape from sodium and chloride retention occurred at both dosage levels while the compound was still being administered This same phenomenon occurs in normal people under continued treatment with desoxycorticosterone The changes that were observed in the hematocrit values and in body weight correlated well with the changes in renal excretion of sodium and chloride *In this subject renal excretion of potassium was not affected significantly* The blood pressure rose mildly during the period when compound B was administered

Loeb What dosage schedule was that?

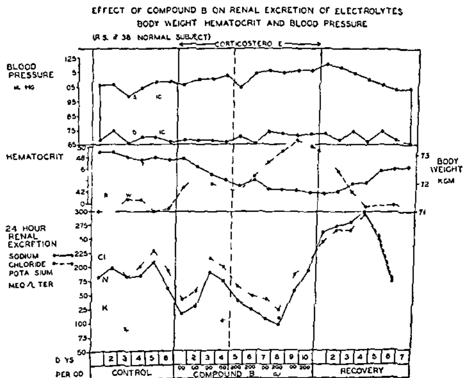


FIGURE 56

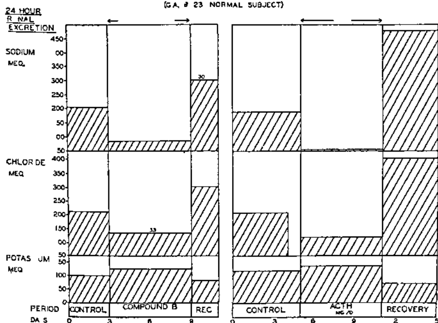

COMPARATIVE EFFECTS IN THE SAME PERSON OF COMPOUND B
AND ACTH ON RENAL EXCRETION OF ELECTROLYTES
(G.A. # 23 NORMAL SUBJECT)
24 HOUR
R NAL
EXCRETION
SODIUM
MEQ.
CHLOR DE
MEQ
POTAS UM
MEQ
PERIOD
DA S
CONTROL
COMPOUND B
REC
CONTROL
ACTH
RECOVERY

FIGURE 59

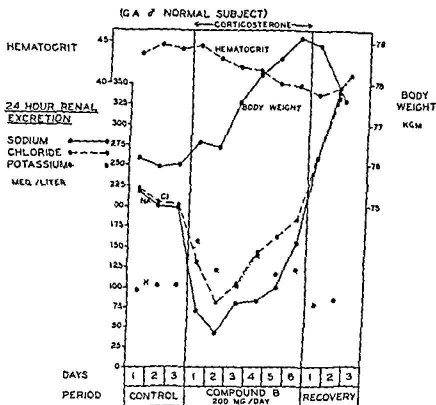


Figure 58

Figure 59 compares in this second normal subject the relative effects upon urinary electrolytes of corticosterone (200 mg per day) and ACTH (100 mg per day) Again ACTH produces the more pronounced effects

Figure 60 indicates the results obtained in both normal subjects with respect to renal excretion of organic elements under the influence of administered compound B Subject G A showed no significant increases in urinary glucose uric acid or nitrogen Subject R S however demonstrated real increases in urinary excretion of glucose and uric acid but there was no effect upon urinary nitrogen So here again we find that there are differences among normal people with respect to the end organ responses So far as the effects of the 11 oxygenated steroids upon organic metabolism are concerned one finds the same situation of course with cortisone One individual given 200 mg per day of

EFFECT OF COMPOUND B COMPOUND E AND ACTH
ON GLUCOSE TOLERANCE

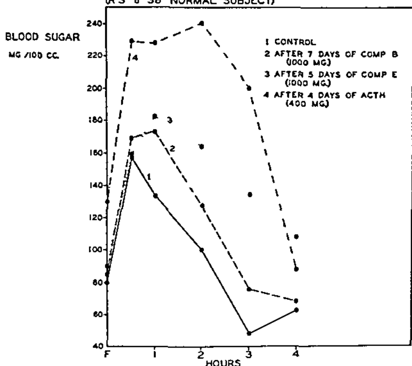


FIGURE 61

cortisone develops frank diabetes while others fail to show such a response

Figure 61 compares the effects upon glucose tolerance in Subject R S of compound B compound E and ACTH *You may recall that* this is the subject whose urinary glucose and uric acid increased on compound B In this subject ACTH produced the greatest impairment of glucose tolerance and the effect of cortisone was also greater in this respect than that of corticosterone

Figure 62 shows that both normal subjects increased their urinary excretions of formaldegenic steroids while receiving large doses of compound B Urinary 17 ketosteroids diminished mildly in Subject G A and remained unchanged in Subject R S

From these observations in the normal subjects one can conclude

(A) That compound B has pronounced effects upon electrolyte metabolism and that these effects are qualitatively similar to those produced by desoxycorticosterone

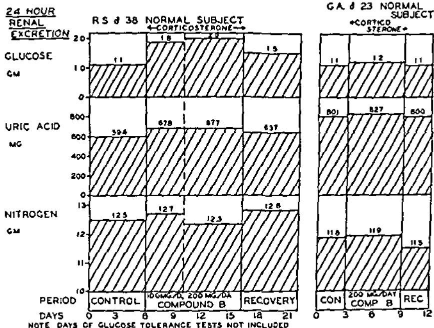

EFFECT OF COMPOUND B ON RENAL EXCRETION
OF GLUCOSE URIC ACID AND NITROGEN
24 HOUR RENAL EXCRETION
RS ♂ 38 NORMAL SUBJECT
←CORTICOSTERONE→
GA ♂ 23 NORMAL SUBJECT
←CORTICOSTERONE→
GLUCOSE
GM
20
10
0
11
18
15
11
12
11
URIC ACID
MG
800
600
400
200
0
594
678
677
637
801
827
800
NITROGEN
GM
13
12
11
10
12.5
12.7
12.3
12.8
11.8
11.9
11.5
PERIOD
CONTROL
100 MG/D, 200 MG/DA
COMPOUND B
RECOVERY
CON
200 MG/DAY
COMP B
REC
DAYS
0 3 6 9 12 15 18 21
0 3 6 9 12
NOTE DAYS OF GLUCOSE TOLERANCE TESTS NOT INCLUDED

FIGURE 60

(B) That compound B exerts mild effects upon organic metabolism which are qualitatively similar to those produced by cortisone

(C) That upon a milligram basis corticosterone has a more intense effect upon inorganic metabolism and a less intense effect upon organic metabolism than does cortisone

Because we wished to demonstrate more critically the effects of compound B upon organic metabolism a patient with coexisting Addison s disease and diabetes mellitus was studied next This patient was studied twice corticosterone having been administered in a dose of 100 mg per day

Figure 63 shows the effects upon renal excretion of electrolytes Retention of sodium and chloride and diuresis of potassium were more intense than in the normal subjects even when the latter received 200 mg per day of compound B

Sayers Was the excretion of potassium less than what you would expect on cortisone administration with an equivalent amount of sodium retention?

Conn I believe not because generally potassium diuresis with corti

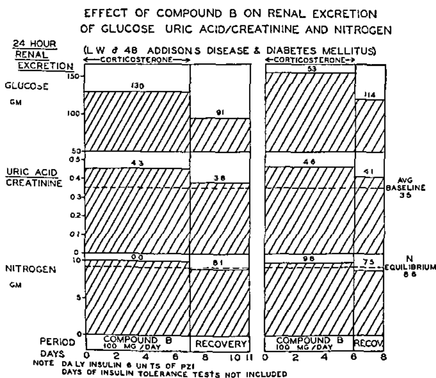


FIGURE 64

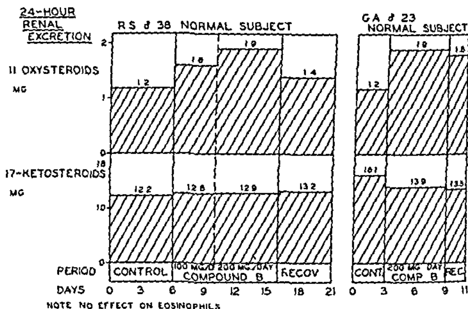


FIGURE 62

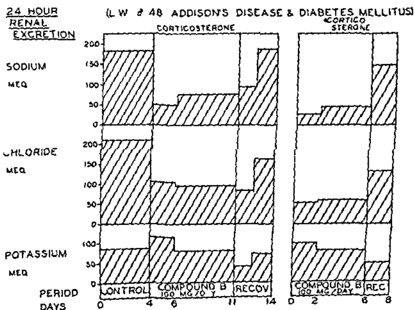


FIGURE 63

Dr Thorn earlier He suggested that if one could push the Addisonian in the direction of diabetes so that he would be hyperglycemic that under these conditions one might be able to demonstrate an anti insulin effect of cortisone and that it is difficult to show an anti insulin effect of cortisone in the Addisonian at his usual level of blood sugar I think that Figure 64 demonstrates this quite well with respect to the anti insulin effect of compound B This effect of compound B was reported years ago in rats by Jensen and Grattan

In a group of experiments upon another case of Addison s disease (Figure 66) we have compared the metabolic effects of 25 mg per day of corticosterone with the effects of a mixture (aqueous suspension) of 20 mg per day of cortisone plus 2 mg per day of desoxycorticosterone Even with this small dose of compound B a sharp desoxycorticosterone like effect is obtained in the Addisonian This effect is more intense than that observed with the cortisone desoxycorticosterone mixture Figure 67 shows that at this level of dosage neither compound B nor the cortisone DCA mixture affected urinary excretion of nitrogen In both experiments however the uric acid-creatinine ratio rose to the same degree

COMPARATIVE EFFECTS OF COMP B AND OF COMP E + DCA (MIXED) UPON RENAL EXCRETION OF ELECTROLYTES

(F H ♂ 43 ADDISON S DISEASE)

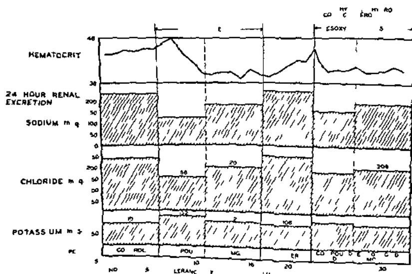


FIGURE 66

sone is less marked Sometimes you do get potassium diuresis on cortisone

Thorn The potassium excretion with cortisone or corticosterone will be the net result of three factors a) the increased renal excretion of potassium, b) increased potassium release from protein breakdown, c) increased potassium deposition with glycogen

Conn The electrolyte effects of compound B resemble those produced by DCA

Figure 64 demonstrates clearly the effects of corticosterone upon organic metabolism in this patient Significant increases were observed in urinary excretion of glucose and nitrogen as well as in the uric acid creatinine ratio In addition Figure 65 indicates that corticosterone exerts an anti insulin effect in confirmation of the early animal experiments of Jensen and Grattan (17) This brings up the point raised by

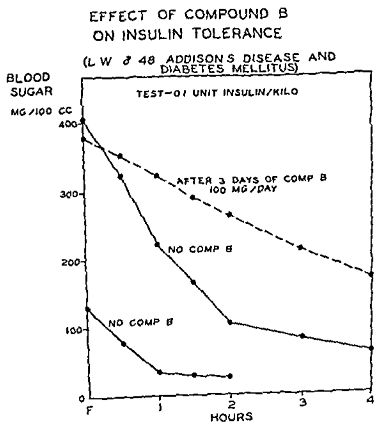


FIGURE 65

TABLE XIV

Comparative Physiological Properties of DCA Compound E and Compound B

	DCA	Corti one (E)	Corticosterone (B)
Electrolyte *Metabolism*	Intense	Mild and Variable	Good
Organic *Metabolism*	None	Intense	Mild
E E C	None	Good	Good
Pep	None	Good	Good
GI Absorption	Small	Good	Good*

Pr l min y

In summary I would like to enumerate a few conclusions that I have drawn from this work

(A) Compound B when administered in untreated Addison s disease corrects the disturbance of electrolyte metabolism Milligram for *milligram the effect of compound B upon electrolyte metabolism is* much less intense than that of DCA giving the advantage of a wider safety range in dosage

(B) *Compound B also corrects the fasting hypoglycemia* which many of these patients exhibit This effect upon organic metabolism although demonstrable in normals is much more evident in adrenal insufficiency

(C) In doses as small as 25 mg per day compound B imparts to the Addisonian a remarkable feeling of well being of pep and of zest for living In this respect it is similar to compound E and both compounds improve the abnormal Addisonian electroencephalogram

(D) Addisonians receiving cortisone alone are frequently improved when *a small amount of DCA is added* daily Compound B alone appears to produce the physiological and metabolic effects of this combination (cortisone plus DCA)

(E) Since DCA is poorly absorbed from the GI tract of man compound B may prove to be the steroid of choice for adequate oral substitution therapy in Addison s disease

(F) Finally it appears that the presence of an oxygen atom on *carbon 11 imparts metabolic activities* which are *responsible for pro*ducing the feeling of well being This particular activity does not

COMPARATIVE EFFECTS OF COMP B AND OF COMP E + DCA (MIXED) UPON URINARY NITROGEN AND URIC ACID-CREATININE RATIO

(F H ♂ 43 ADDISON S DISEASE)

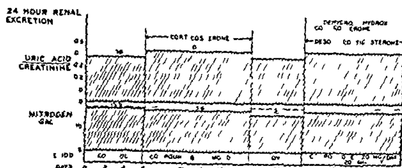


FIGURE 67

PREVENTION OF FASTING HYPOGLYCEMIA WITH CORTICOSTERONE

(A H ♀ 38, ADDISON S DISEASE)

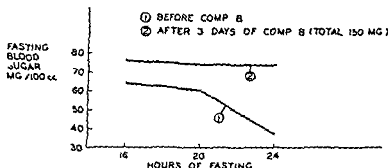


FIGURE 68

Figure 68 indicates that the fasting hypoglycemia which is observed in some cases of Addison s disease can be prevented by the administration of corticosterone

Table XIV is intended to portray a rough comparison of the physiological properties of DCA cortisone and corticosterone as they relate to the untreated Addisonian A remark is required regarding the property which we have labeled as pep Although this is based wholly upon clinical observation it is nonetheless definite It consists of an intangible feeling of well being and of zest for activity and life It is a property which we have all observed during administration of either ACTH or cortisone It is difficult to assign relative values with respect to a property as nebulous as this but corticosterone possesses it

during fasting and one does not observe it, to the same degree if the animal is well fed

Conn That may be the reason why the diabetic went into negative nitrogen balance on compound B while the other subjects did not

Long In a certain sense the diabetic is already fasting

Loeb It may be of interest to point out when we gave 18 5 mg of corticosterone a day that was superimposed upon a maintenance dose of DCA

Pincus In our studies of the steroid in the isolated adrenal perfused with ACTH corticosterone was produced in about the same amount as compound F these being the two major alpha ketols (19) In regard to the point that you brought up before there is therefore a very good likelihood that this hormone does represent the natural secretory product of the adrenal cortex

Ingle When Reichstein isolated corticosterone (20) he believed that it was the hormone of the adrenal cortex and this was the reason for assigning to it the name corticosterone It was the first pure compound which easily maintained life in adrenalectomized animals This had been done earlier with cortisone but large amounts were required

Long I believe the studies of Samuels and his colleagues (21) on the composition of steroids in the adrenal vein blood of the dog indicate that compound F and corticosterone are the major constituents Is that right?

Sayers Right

Pincus Reported also by Zaffaroni (22)

Thorn There would be considerable advantage for the patient if corticosterone or dehydrocorticosterone were found to be active by mouth since they could replace the combination of cortisone and desoxycorticosterone for daily maintenance

Conn I failed to say that we now have had two cases of Addison s disease on 25 mg a day of corticosterone intramuscularly for sixty days These patients have been eating ravenously gaining weight without edema and have been maintained in excellent health One preparation that was only 80 per cent pure was given orally by Robinson in large doses and produced marked edema Dr Ingle has done work in rats I believe indicating good absorption Is that right?

Ingle It is quite active orally

Long I can confirm Dr Ingle on that point because a good many years ago he showed you that you could put the corticosterone in the drinking water of the rat and obtain marked effects

Ingle Dr Conn noted the lack of correlation between the known biochemical effects of adrenal cortical hormones and their effect on the pep of the patient Our experience in animals is exactly comparable

parallel any of the metabolic changes which we have been able to measure

Ralli Is cortisone given orally just as effective as when injected in the Addisonian?

Thorn Cortisone by mouth is a very effective means of treating patients with Addison s disease The dose is the same as by injection i e , 12 5 to 25 mg daily

Conn How frequently are you giving it during the day?

Thorn At the present time we give cortisone every eight to twelve hours It is quite probable that the rapid action following orally ad ministered cortisone will tend to decrease the overdosage effect which is so easily obtained with prolonged administration of desoxycortico sterone acetate given intramuscularly

Selye What is the particular significance of the eosinophils in the treated Addisonian?

Thorn The return of the eosinophils to normal suggests that the effect of cortisone is wearing off One cannot of course say that other metabolic aspects of cortisone action follow a similar time pattern

Long Have you had the opportunity to try compound B in rheuma toid arthritis?

Conn Dr Robinson who runs our Arthritis Clinic has tried it in one patient At a level of 200 mg per day of corticosterone there was very little or no antirheumatic activity

Thorn I would like to state that our experience with dehydro corticosterone in roughly the same dosage as the compound A has been similar to Dr Conn s with compound B Unfortunately we could not get Mercks to prepare anymore We felt that it had distinct use fulness in patients with Addison s disease and three of our most severe patients were well maintained on 3 mg a day These studies have all been summarized in our paper Metabolic Changes Induced by Syn thetic 11 dehydrocorticosterone (18)

Selye We did a good deal of work with dehydrocorticosterone on rats and found that 2 5 mg per day has a very pronounced growth inhibiting effect in young animals It also prevents the formation of the topical irritation arthritis produced with formalin and the anaphy lactic reaction to egg white

Long Dr Conn did you make any observations on the nitrogen excretion during fasting?

Conn We have not done that

Long I think that this should be borne in mind I am sure you are aware that if the individual is receiving adequate calories he is going to protect or preserve his own tissue proteins Consequently the catabolic effect on protein metabolism of any of these steroids is most marked

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We like to refer to the vim and vigor effect of these hormones which we are as yet unable to define in terms of biochemistry Our criterion of vigor in the rat is its ability to work The effect of the cortical hormones upon the ability of adrenalectomized animals to work can be dissociated from the known effects upon electrolyte and organic metabolism

Long Are there any further questions? If not I should like to express on your behalf our appreciation to Dr Fremont Smith and Miss Freed for getting this meeting together and also to thank them for the very pleasant circumstances under which we have met

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